Prostaglandin $F_{2\alpha}$-stimulated phospholipase D activation in osteoblast-like MC3T3-E1 cells: involvement in sustained 1,2-diacylglycerol production

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In [^{3}H]myristic acid-labelled osteoblast-like MC3T3-E1 cells, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced PLD activity was assessed by measuring the [^{3}H]phosphatidylethanol (PEt) formation in the presence of ethanol. Inhibition of the increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by U73122, an inhibitor of phosphoinositide-specific phospholipase C (PI-PLC), or chelation of extracellular Ca^{2+} with EGTA or of intracellular Ca^{2+} with BAPTA, suppressed $PGF_{2\alpha}$-induced phospholipase D (PLD) activation. Neither protein kinase C (PKC) inhibitors nor PKC down-regulation with phorbol 12-myristate 13-acetate affected $PGF_{2\alpha}$-induced [^{3}H]PEt formation. In permeabilized cells, guanosine 5′-[γ-thio]triphosphate enhanced $PGF_{2\alpha}$'s potency in [^{3}H]PEt formation in the presence of Ca^{2+}. The pretreatment of intact cells with pertussis toxin failed to inhibit $PGF_{2\alpha}$-induced [^{3}H]PEt formation. $PGF_{2\alpha}$ caused a biphasic production of [^{3}H]1,2-diacylglycerol ([^{3}H]1,2-DAG) in [^{3}H]glycerol-labelled cells. The initial transient phase was decreased by U73122, whereas the late sustained phase was decreased by ethanol and the phosphatidic acid phosphohydrolase inhibitor, propranolol. From these results, it was suggested that $PGF_{2\alpha}$-induced PLD activation was mediated by the dual control of the $[Ca^{2+}]_i$ increase due to PI-PLC activation and activation of pertussis-toxin-insensitive G-protein, but not mediated by PKC, and also that PLD activation was involved in the late sustained 1,2-DAG generation in MC3T3-E1 cells.

INTRODUCTION

There has been substantial evidence for the agonist-induced activation of phospholipase D (PLD; EC 3.1.4.4) in a variety of mammalian cells [1,2]. This enzyme catalyses not only the hydrolysis of phospholipids, producing phosphatidic acid (PA) and the free polar group, but also the transphosphatidylation reaction [3]. In the presence of ethanol, the activity of PLD specifically produces phosphatidylethanol (PEt) by the latter reaction [4]. It was shown by measuring reaction products that PLD activation caused by the agonist(s), e.g. hormones, growth factors and cytokines, was associated with activation of protein kinase C (PKC) [5–7], increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) [8–11] and/or increase in GTP-binding protein (G-protein) [11,12]. In fact, in several types of cells PLD is activated by the PKC activator phorbol 12-myristate 13-acetate (PMA) [13], Ca^{2+} ionophore [8,14] or GTP analogue [14,15], and inhibited by PKC down-regulation [16,17], PKC inhibitors (H-7 [18], staurosporine [19], Ro-31-8220 [17]), the intracellular-Ca^{2+} chelator BAPTA [8], the phosphoinositide-specific phospholipase C (PI-PLC) inhibitor U73122 [8] and inactive analogues of guanine nucleotides (GDP or its β-thio analogue [14,15]). Furthermore, in many cases of the agonist-induced PLD activation, it was suggested that the PLD activation occurred downstream of PI-PLC activation [8,20–22]. However, the PKC-independent pathway for PLD activation was also observed to operate in some cell types [6,23].

Prostaglandins (PGs) are important local factor(s) not only in bone resorption but also its formation [24–26] by both paracrine and autocrine mechanism(s). It was suggested by previous investigators that some effects of PG(s) were mediated by the activation of adenylate cyclase, and others were mediated by the stimulation of PtdIns(4,5)P_2 hydrolysis by PLC [25,27,28]. $PGF^{2\alpha}$, a potent proliferating agonist for osteoblasts, causes activation of PI-PLC leading to increased $[Ca^{2+}]_i$ and PKC activation in some cloned cells [28–31], including osteoblast-like MC3T3-E1 cells [25,32].

Recently, in addition to 1,2-diacylglycerol (1,2-DAG) produced by PtdIns(4,5)P_2 hydrolysis, other sources of 1,2-DAG are proposed, i.e. phosphatidylcholine (PC) hydrolysis by PLD and/or PLC [22,33–37]. PLD activation results in the production of PA, which is then dephosphorylated by PA phosphohydrolase (EC 3.1.3.4) to produce 1,2-DAG. Such PC-derived 1,2-DAG produces a sustained phase of agonist-induced 1,2-DAG formation. Prolonged 1,2-DAG formation [29] and PKC activation [30] were observed in fibroblasts stimulated by $PGF_{2\alpha}$, indicating the presence of cross-talk between PLD and PI-PLC pathways [8]. In osteoblast(-like) cells, activation of PtdIns(4,5)P_2 hydrolysis by PLC [27], PKC activation and/or increase in $[Ca^{2+}]_i$ [28] were elicited by hormones, growth factors and cytokines, including PGs [25,27]. But there is no report of agonist-induced PLD activation in osteoblastic cells.

In the present study, we have investigated the activation of PLD, its regulatory mechanism and the involvement of the enzyme in 1,2-DAG production in $PGF_{2\alpha}$-stimulated osteoblast-like MC3T3-E1 cells.

Abbreviations used: α-MEM, α-Modified Eagle's Minimum Essential Medium; BAPTA, 1,2-bis-(2-aminophenoxy)ethane-*NNN'N'*-tetra-acetic acid; BAPTA/AM, tetra-acetoxymethyl ester of BAPTA; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concn.; FBS, fetal-bovine serum; fura-2, 1-[2-(5′-carboxyoxazol-2′-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*NNN'N'*-tetra-acetic acid; fura-2/AM, penta-acetoxymethyl ester of fura-2; GTP[S], guanosine 5′-[γ-thio]triphosphate; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride; PA, phosphatidic acid; PC, phosphatidylcholine; PEt, phosphatidylethanol; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PI-PLC, phosphoinositide-specific phospholipase C; PLD, phospholipase D; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; U73122, 1-(6-{[17β-3-methoxyoestra-1,3,5(10)-trien-17-yl]amino}hexyl)-1*H*-pyrrole-2,5-dione; 1,2-DAG, 1,2-diacylglycerol.

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MATERIALS AND METHODS

Materials

$PGF_{2\alpha}$ was kindly supplied from Ono Pharmaceuticals Co. (Osaka, Japan). PMA, ionomycin, propranolol, thapsigargin and BSA (fraction V) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The acetoxymethyl esters of fura-2 (fura-2/AM) and BAPTA (BAPTA/AM) (full systematic names given in title-page footnote) were obtained from Dojin Laboratories (Kumamoto, Japan). 1-({6[17β-3-Methoxyoestra-1,3,5(10)-trien-17-yl]amino}hexyl)-1*H*-pyrrole-2,5-dione (U73122) was generously given by Dr. John E. Bleasdale, The Upjohn Co. (Kalamazoo, MI, U.S.A.). 1-(5-Isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H-7) was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Calphostin C and K-252a were purchased from Kyouwa Hakkou Co. (Tokyo, Japan). α-Modified Eagle's Minimum Essential Medium (α-MEM) and fetal bovine serum (FBS) were obtained from Gibco Laboratories (Grand Island, MY, U.S.A.) and Flow Laboratories (Dublin, VA, U.S.A.) respectively. [1(3)-^{3}H]Glycerol (37–110 GBq/mmol) and [9,10(n)-^{3}H]myristic acid (1.5–2.2 TBq/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.). All other reagents used were of analytical grade.

Cell culture

Cloned MC3T3-E1 cells were kindly supplied by Dr. H. Kodama (Ohou University, Japan). The cells were cultured in α-MEM supplemented with 10% FBS at 37 °C under an atmosphere of 5% CO_2 in air. Cells were subcultured every 3 days as described by Kodama et al. [38].

[^{3}H]PEt formation in intact MC3T3-E1 cells

PLD activity was determined as previously described [10,23,39] by measuring [^{3}H]PEt produced via PLD-catalysed transphosphatidylation [20] in [^{3}H]myristic acid-labelled cells. MC3T3-E1 cells were plated at a density of 2×10^4 cells/well on 6-well plates and cultured for 4 days. For metabolic labelling of phospholipids, cells were cultured for 12 h in 0.75 ml of α-MEM + 0.3% BSA containing 1 μCi of [^{3}H]myristic acid/well. Cells were washed twice in 1 ml of Ca^{2+}/Mg^{2+}-free PBS, and then preincubated in Hepes buffer (25 mM NaHepes, pH 7.4, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose and 0.1% BSA) containing ethanol (1%, v/v) at 37 °C for 5 min with or without the indicated reagents. After preincubation, cells were stimulated with $PGF_{2\alpha}$ (10 pM–10 μM), ionomycin (2 μM), A23187 (1 μM) or thapsigargin (1 μM) in 1 ml of Hepes buffer containing ethanol (1%) for the indicated times. The reaction was then terminated by addition of 1.3 ml of ice-cold methanol, and lipids were then extracted by the method of Bligh and Dyer [40]. [^{3}H]PEt was separated from other phospholipids by two-dimensional t.l.c. on silica gel 60 plates. The first solvent system was chloroform/methanol/28% NH_3 (65:35:6, by vol.) and the second was chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, by vol.). The area corresponding to PEt was scraped off the plate and radioactivity was measured in liquid-scintillation counter.

[^{3}H]PEt formation in digitonin-permeabilized MC3T3-E1 cells

MC3T3-E1 cells were grown and labelled with [^{3}H]myristic acid in 6-well plates as described above. The cells were washed in 1 ml of Ca^{2+}/Mg^{2+}-free PBS twice, and then incubated in potassium glutamate buffer (20 mM Pipes, pH 6.9, 139 mM potassium glutamate, 1 mM $MgCl_2$, 1 mM Mg-ATP and 5 mM EGTA) containing 10 μM digitonin at 25 °C for 15 min as previously described [23,41]. Incubations were started by replacing the solution with 1 ml of potassium glutamate buffer containing the indicated concentrations of free Ca^{2+} buffered with 1 mM $CaCl_2$/5 mM EGTA in the presence of 1% ethanol and the test reagents {i.e. guanosine 5′-[γ-thio]triphosphate (GTP[S]), GDP, $PGF_{2\alpha}$ etc.} at various concentrations for the indicated times. Incubations were terminated as described above and lipids were extracted by the method of Bligh and Dyer [40]. Then [^{3}H]PEt was separated by two-dimensional t.l.c. for radioactivity measurement.

$PGF_{2\alpha}$-induced [^{3}H]1,2-DAG formation in intact MC3T3-E1 cells

For metabolically labelling phospholipids, the cells were cultured in 0.75 ml of α-MEM + 0.3% BSA containing 2 μCi of [^{3}H]glycerol/well for 36 h. The cells were washed twice, and then incubations were started by replacing the buffer with 1 ml of Hepes buffer containing the test reagents at various concentrations and for the indicated times. Incubations were terminated by addition of ice-cold methanol, and lipids were extracted by the method of Bligh and Dyer [40]. [^{3}H]1,2-DAG was separated by t.l.c. on silica gel 60 plates (pretreated with 0.4 M borate) in the solvent system chloroform/acetone (24:1, v/v).

Measurement of $[Ca^{2+}]_i$ in single cells

The cells were plated at a density of 1×10^3 cells/well of the Flexiperm-Disc (Heraeus Biotechnologie, Hanau, Germany) in α-MEM containing 10% FBS and cultured for 72 h. Then the cells were labelled with fura-2/AM, and $[Ca^{2+}]_i$ changes by each reagent were measured for single cells by using a fluorescence image analyser (ARGUS-100/CA; Hamamatsu Photonics Corp, Hamamatsu, Japan) as previously described [42].

RESULTS

$PGF_{2\alpha}$-induced PLD activation in MC3T3-E1 cells

To investigate PLD activity, we measured the accumulation of [^{3}H]PEt produced by transphosphatidylation activity in the presence of ethanol. [^{3}H]Myristic acid was observed to be incorporated to a large extent into PC of intact MC3T3-E1 cells, up to over 70% during incubation for 36 h (Table 1). The [^{3}H]myristic acid-labelled cells were washed and preincubated for 5 min with Hepes buffer containing 1% ethanol, and then stimulated. [^{3}H]PEt was increased by addition of 1 μM $PGF_{2\alpha}$, reaching a plateau by about 2 min (Figure 1). The $PGF_{2\alpha}$-induced $[Ca^{2+}]_i$ increase (insert in Figure 1) was concurrent with an initial [^{3}H]PEt increase. For a 5 min incubation, $PGF_{2\alpha}$ (1 nM to 1 μM) produced an increase in [^{3}H]PEt in a dose-dependent fashion (results not shown). The profile for [^{3}H]PEt formation was similar to that for the $[Ca^{2+}]_i$ increase (T. Sakai and T. Sugiyama, unpublished work) and inositol phosphate(s) production previously described in MC3T3-E1 cells [27].

Role of PI-PLC, $[Ca^{2+}]_i$ increase and PKC in $PGF_{2\alpha}$-induced PLD activation

In some cell types, including MC3T3-E1 cells, $PGF_{2\alpha}$ was observed to activate PtdIns(4,5)P_2 hydrolysis, which in turn caused an increase in $[Ca^{2+}]_i$ and activation of PKC [28–31]. The agonist-induced PI-PLC activation occurring before PLD activation has been reported in many studies [5,7–10,16,17,43]. If

Table 1 Distribution of [^{3}H]myristic acid radioactivity in major phospholipids and 1,2-DAG of MC3T3-E1 cells

MC3T3-E1 cells were incubated with [^{3}H]myristic acid (1 μCi/well for 12 h), then cells were washed twice with Ca^{2+}/Mg^{2+}-free PBS and incubated in Hepes buffer with or without 1 μM $PGF_{2\alpha}$ in 1% ethanol for 5 min. Total cellular lipids were extracted and analysed by t.l.c. as described in the Materials and methods section. Total radioactivity of [^{3}H]myristic acid-labelled lipid (per culture well) was 685490 ± 49315 d.p.m. Data represent means ± S.D. ($n = 3$): *significantly different from the control value ($P < 0.01$). Abbreviation: PE, phosphatidylethanolamine.

	[^{3}H]Myristic acid incorporation		
	Control		1 μM $PGF_{2\alpha}$
Cellular lipids	Radioactivity (d.p.m.)	(% of total)	(% of total)
PA	3523 ± 644	0.51 ± 0.02	1.11 ± 0.12*
PC	517550 ± 23650	75.5 ± 3.45	72.1 ± 4.6
PE	47234 ± 5756	6.91 ± 0.13	5.94 ± 0.22*
PEt	3427 ± 910	0.49 ± 0.12	1.03 ± 0.04*
Inositol plus serine phospholipids	27776 ± 3705	4.06 ± 0.03	3.17 ± 0.33*
Lyso-PC	6032 ± 206	0.88 ± 0.03	0.85 ± 0.01
Sphingomyelin	62791 ± 6375	9.16 ± 0.93	10.49 ± 0.47
1,2-DAG	4891 ± 177	0.83 ± 0.03	2.73 ± 0.28*

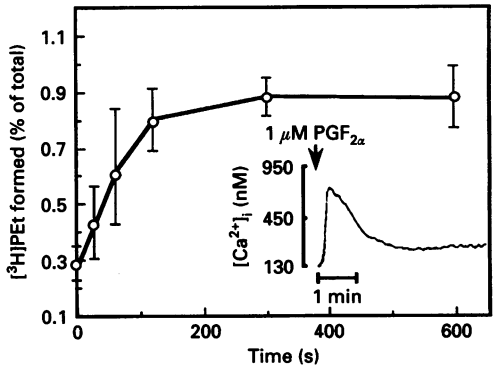

Figure 1 Time course of production of [^{3}H]PEt in response to $PGF_{2\alpha}$ in MC3T3-E1 cells

[^{3}H]Myristic acid-labelled MC3T3-E1 cells were stimulated for the indicated times with 1 μM $PGF_{2\alpha}$ in 1% ethanol. The lipid samples were then analysed by two-dimensional t.l.c. for [^{3}H]PEt as described in the Materials and methods section. The radioactivity in the controls was: PC, 428387 d.p.m.; PEt, 2617 d.p.m. Data are means ± S.E.M. from three experiments. The insert shows a trace of the $PGF_{2\alpha}$-induced $[Ca^{2+}]_i$ response in fura-2-labelled MC3T3-E1 cells. The cells were loaded as described in the Materials and methods section and stimulated with 1 μM $PGF_{2\alpha}$ at the arrow. $[Ca^{2+}]_i$ was calculated with a fluorescence image analyser ARGUS-100/CA.

Table 2 Involvement of PI-PLC and $[Ca^{2+}]_i$ increase in $PGF_{2\alpha}$-induced PLD activation in MC3T3-E1 cells

For measurement of [^{3}H]PEt accumulation, [^{3}H]myristic acid-labelled MC3T3-E1 cells were washed twice with Ca^{2+}/Mg^{2+}-free PBS, then preincubated in Hepes buffer with or without 2 μM U73122 or 10 μM BAPTA/AM for 20 or 60 min respectively. During the last 5 min of the treatment, the buffer was changed to 1% ethanol-supplemented buffer with or without (EGTA and BAPTA/AM) 1 mM $CaCl_2$, and stimulated with each agonist for 5 min. The reactions were then stopped by addition of ice-cold methanol, and the lipid samples were analysed by two-dimensional t.l.c. for [^{3}H]PEt as described in the Materials and methods section. The radioactivity in the controls was: PC, 500313 d.p.m.; PEt, 2469 d.p.m. For measurement of $[Ca^{2+}]_i$, the cells were plated at a density of 1×10^3 cells/well on the Flexiperm-Disc. After culture for 72 h, the cells were loaded with fura-2/AM (in some samples, 2 μM U73122 or 10 μM BAPTA/AM was added and incubated for 20 or 60 min respectively), and then the $[Ca^{2+}]_i$ change was monitored and calculated with an image analyser ARGUS-100/CA. The values express the peak $[Ca^{2+}]_i$ increase by $PGF_{2\alpha}$. Data are means ± S.D. for n experiments. The pretreatment with U73122 or EGTA gave no significant changes in basal $[Ca^{2+}]_i$ (control, 115.2 ± 35.2 nM; U73122, 138 ± 32.4 nM; EGTA, 131 ± 45.8 nM), and BAPTA/AM decreased basal $[Ca^{2+}]_i$ (73 ± 44.4 nM). Abbreviation: N.D., not determined.

	$[Ca^{2+}]_i$ Increase (% of basal)	[^{3}H]PEt formed (% of basal)
Control (1 μM $PGF_{2\alpha}$)	384 ± 145 ($n = 23$)†	353 ± 104 ($n = 22$)†
Pretreated with		
1 μM U73122	134 ± 59 ($n = 20$)*	183 ± 32 ($n = 5$)†
0.5 mM EGTA	187 ± 101 ($n = 25$)*	185 ± 27 ($n = 8$)†
10 μM BAPTA/AM	109 ± 23 ($n = 7$)*	169 ± 28 ($n = 5$)†
2 μM Ionomycin	418 ± 57 ($n = 7$)†	161 ± 85 ($n = 14$)*
1 μM A23187	N.D.	131 ± 26 ($n = 3$)*
1 μM Thapsigargin	453 ± 70 ($n = 6$)†	107 ± 31 ($n = 8$)*

* Significantly different from the control value ($P < 0.01$).
† Significantly different from the basal value ($P < 0.01$).

$PGF_{2\alpha}$-induced PLD activation is secondary to PI-PLC activation, then it would be expected that prevention of $PGF_{2\alpha}$-induced PI-PLC activation inhibits PLD activation. As shown in Table 2, pretreatment for 20 min with the PLC inhibitor U73122 [8,44] markedly decreased not only the $[Ca^{2+}]_i$ increase $PGF_{2\alpha}$-induced but also the $PGF_{2\alpha}$-induced [^{3}H]PEt formation. These results indicate that inhibition of $PGF_{2\alpha}$-induced PI-PLC activation leads to decreased PLD activation, as reported in other cells [8].

Then we examined how PtdIns(4,5)P_2 hydrolysis is coupled to PLD activation. Some investigators [8–10,34] have proposed that the $[Ca^{2+}]_i$ increase resulting from PI-PLC activation is required for PLD activation, and others [16–19,34] reported involvement of PKC after PI-PLC activation. To examine the role of the $[Ca^{2+}]_i$ increase, we investigated the effect of the prevented $[Ca^{2+}]_i$ increase and also the agonist-independent $[Ca^{2+}]_i$ increase on $PGF_{2\alpha}$-induced PLD activation. As shown in Table 2, chelation of extracellular Ca^{2+} (no added Ca^{2+} and in the presence of 0.5 mM EGTA) and intracellular Ca^{2+} (10 μM BAPTA/AM) diminished $PGF_{2\alpha}$-induced $[Ca^{2+}]_i$ increases in fura-2-loaded single cells and markedly inhibited PLD activation. Therefore the $[Ca^{2+}]_i$ rise was thought to play a crucial role in $PGF_{2\alpha}$-induced PLD activation. In order to ascertain further the implication of $[Ca^{2+}]_i$ in PLD activation, we examined whether or not receptor-independent $[Ca^{2+}]_i$ increases could mimic the $PGF_{2\alpha}$-induced PLD activation. The $[Ca^{2+}]_i$ increases caused by

Table 3 Effects of PKC inhibitors and PKC down-regulation on $PGF_{2\alpha}$-induced PLD activation in MC3T3-E1 cells

For PKC down-regulation, [^{3}H]myristic acid-labelled MC3T3-E1 cells were preincubated with 330 nM PMA for 24 h, and for PKC inhibition the cells were preincubated for 20 min in Hepes buffer with PKC inhibitors. During the last 5 min of the treatment, the buffer was changed to 1%-ethanol-supplemented, and stimulated with 1 μM $PGF_{2\alpha}$ for 5 min. The reactions were then stopped by addition of ice-cold methanol, and the lipid samples were analysed by two-dimensional t.l.c. for [^{3}H]PEt as described in the Materials and methods section. The radioactivity in the controls was: PC, 553580 d.p.m.; PEt, 2108 d.p.m. Data are means ± S.D. for n experiments.

	[^{3}H]PEt formed (% of basal)
Control (1 μM $PGF_{2\alpha}$, 2 min)	318 ± 104 (n = 15)
Pretreated with	
H-7 (50 μM)	407 ± 56 (n = 6)
Calphostin C (2 μM)	451 ± 173 (n = 5)
K-252a (1 μM)	446 ± 58 (n = 3)
Staurosporine (0.1 μM)	287 ± 23 (n = 3)
PKC-down-regulated (330 nM PMA, 24 h)	250 ± 43 (n = 7)

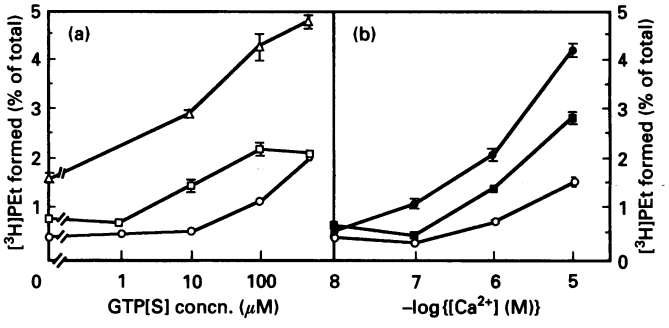

Figure 2 Synergistic activation of PLD by Ca^{2+} and GTP[S] in permeabilized MC3T3-E1 cells

[^{3}H]Myristic acid-labelled MC3T3-E1 cells were permeabilized with 10 μM digitonin at 25 °C for 15 min as described in the Materials and methods section, then incubated with the indicated concentrations of GTP[S] in the presence of Ca^{2+}–EGTA-buffered solution containing 1% ethanol at 37 °C for 30 min. (**a**) ○, pCa7; □, pCa6; △, pCa5; (**b**) ■, 10 μM GTP[S]; ●, 100 μM GTP[S]; ○, 0 μM GTP[S]. The radioactivity in the controls (pCa8; 0 μM GTP[S]) was: PC, 507761 d.p.m.; PEt, 2807 d.p.m. Data are means ± S.E.M. from two experiments.

ionomycin and thapsigargin were larger than that with $PGF_{2\alpha}$ stimulation. However, [^{3}H]PEt formation was much less with either treatment, compared with $PGF_{2\alpha}$-induced PEt production. This suggested that the Ca^{2+} increase was needed, but it alone was not sufficient, for $PGF_{2\alpha}$-induced PLD activation, and additional factor(s) is required for its full activation. A likely candidate is PKC activated via PtdIns(4,5)P_2 hydrolysis. Thus [^{3}H]PEt formation was examined in cells pretreated with PKC inhibitors or exposed to long-term PMA treatment to down-regulate PKC activity. Although a PKC inhibitor, H-7, inhibited PKC activity in MC3T3-E1 cells [42], $PGF_{2\alpha}$-induced [^{3}H]PEt formation was unaffected by this inhibitor. Other PKC inhibitors, K-252a [45], calphostin C [46], and staurosporine, also did not decrease the [^{3}H]PEt production (Table 3). In cells with PKC down-regulated by incubation with 330 nM PMA for 24 h [42], there was apparently no significant inhibitory effect on $PGF_{2\alpha}$-induced PLD activation.

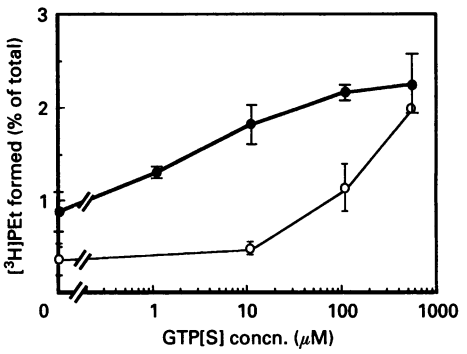

Figure 3 Effect of GTP[S] on $PGF_{2\alpha}$-induced PLD activation in permeabilized MC3T3-E1 cells

[^{3}H]Myristic acid-labelled MC3T3-E1 cells were permeabilized with 10 μM digitonin at 25 °C for 15 min as described in the Materials and methods section, then incubated with the indicated concentration of GTP[S] with (●) or without (○) 1 μM $PGF_{2\alpha}$ at pCa7 containing 1% ethanol at 37 °C for 30 min. The radioactivity in the controls was: PC, 488288 d.p.m.; PEt, 1666 d.p.m. Data are means ± S.E.M. from two experiments.

Effects of guanosine phosphate analogues on $PGF_{2\alpha}$-induced PLD activation in permeabilized cells

Several studies have reported the implication of G-protein in agonist-induced PLD activation in some types of cells [11,12,14,15]. In our preliminary experiment, NaF, a potent G-protein-activating factor, stimulated the PLD activity as observed in $PGF_{2\alpha}$-induced intact cells (results not shown). As the G-protein-activating factors NaF and the unhydrolysable GTP analogue, GTP[S], also stimulated PI-PLC [47] in these cells, which caused a $[Ca^{2+}]_i$ change, we investigated the roles of G-protein in $PGF_{2\alpha}$-induced PLD activation in the permeabilized cells. In a $[Ca^{2+}]_i$-buffered condition, addition of GTP[S] to cells led to a significant dose-dependent increase in [^{3}H]PEt formation in the presence of 1% ethanol and physiological concentration of Ca^{2+} (Figure 2). Further, the activation of PLD activity was Ca^{2+}-dependent, and Ca^{2+} alone in the absence of GTP[S] produced an increase in [^{3}H]PEt level, but significantly lower than in its presence. At 10 μM and 100 μM GTP[S], the PLD activity was enhanced at physiological Ca^{2+} concentration in a dose-dependent manner (Figure 2b). These data indicated that Ca^{2+} and GTP[S] exert a synergistic action on PLD activation. In the presence of 100 nM Ca^{2+}, which is the $[Ca^{2+}]_i$ level in the resting cells, $PGF_{2\alpha}$-mediated PLD activation was enhanced by addition of GTP[S] in the medium (Figure 3). Increase of Ca^{2+} to 1 μM, which is nearly equivalent to the $PGF_{2\alpha}$-induced $[Ca^{2+}]_i$ level, shifted the plot of [^{3}H]PEt formation to the left (results not shown). To ascertain the involvement of G-protein, we used GDP, which is known to inhibit the PLD activity induced by GTP[S] [14,15]. As shown in Figure 4, the PLD activation caused by 100 μM GTP[S] was considerably decreased by addition of 1 mM GDP. The inhibitory effect of GDP was also observed in $PGF_{2\alpha}$-stimulated cells, but its extent was smaller. Pretreatment of intact MC3T3-E1 cells with pertussis toxin (300 ng/ml) for 12 h failed to suppress the PLD activity by $PGF_{2\alpha}$ (369 ± 196% increase from basal; mean ± S.D., n = 4). Under this condition, the α subunit(s) of the pertussis-toxin substrate(s) was ADP-ribosylated, as described in our previous report [47]. These results suggest that pertussis-toxin-insensitive G-protein may be involved in $PGF_{2\alpha}$-induced PLD activation.

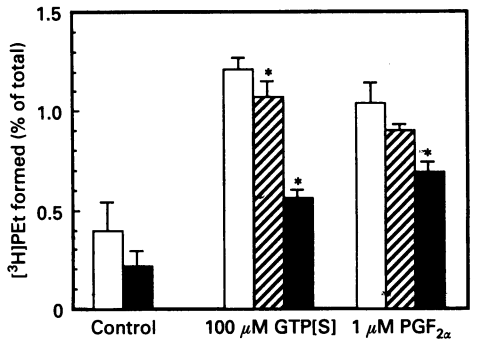

Figure 4 Inhibitory effect of GDP on GTP[S]- and $PGF_{2\alpha}$-induced PLD activation in permeabilized MC3T3-E1 cells

[^{3}H]Myristic acid-labelled MC3T3-E1 cells were permeabilized as described in the Materials and methods section, then stimulated with 100 μM GTP[S] or 1 μM $PGF_{2\alpha}$ in the presence of indicated concentrations of GDP at pCa7 containing 1% ethanol at 37 °C for 30 min. □, No GDP (controls); ▨, with 100 mM GDP; ■, with 1 mM GDP. Data are means ± S.E.M. from two experiments: *significant difference from non-treated cells ($P < 0.05$).

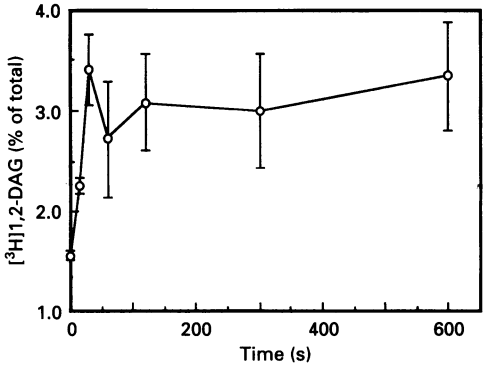

Figure 5 Time course of $PGF_{2\alpha}$-stimulated [^{3}H]1,2-DAG formation in MC3T3-E1 cells

[^{3}H]Glycerol-labelled MC3T3-E1 cells were stimulated for the indicated times with 1 μM $PGF_{2\alpha}$. [^{3}H]1,2-DAG was separated by t.l.c. in the solvent system chloroform/acetone (24:1, v/v). Data are means ± S.E.M. from three experiments. The radioactivity in total lipids was 315751 ± 35790 d.p.m./well, and the distribution in other lipids at 0 s was: PA, 6.29 ± 0.73%; PC, 47.22 ± 0.77%; inositol plus serine phospholipids, 13.07 ± 0.08%; phosphatidylethanolamine, 14.67 ± 0.87%; lyso-PC, 1.00 ± 0.03%; 1,2-DAG, 1.56 ± 0.2%.

Role of PLD in $PGF_{2\alpha}$-induced sustained 1,2-DAG formation

We have examined the role of PLD activation by $PGF_{2\alpha}$ in the sustained production of 1,2-DAG. Figure 5 shows the time course of $PGF_{2\alpha}$-induced 1,2-DAG formation in MC3T3-E1 cells. When the cells prelabelled with [^{3}H]glycerol for 36 h were stimulated by 1 μM $PGF_{2\alpha}$, a supramaximal concentration for activation of both PI-PLC [27,28] and PLD, the increase in 1,2-DAG was biphasic. The early phase of 1,2-DAG increase, to a peaking at 30 s, was concurrent with the $[Ca^{2+}]_i$ increase (see Figure 1) elicited by PI-PLC, and this initial phase was followed by a sustained increase in 1,2-DAG, which reached a plateau after 2 min and then remained elevated for the period examined (10 min). To ascertain that PLD activity acts in such $PGF_{2\alpha}$-induced 1,2-DAG production, we have examined the effects of

Table 4 Role of PLD activation in $PGF_{2\alpha}$-induced [^{3}H]1,2-DAG formation in MC3T3-E1 cells

[^{3}H]Glycerol-labelled MC3T3-E1 cells were stimulated with 1 μM $PGF_{2\alpha}$ for 30 s or 5 min with or without ethanol, propranolol or U73122. [^{3}H]1,2-DAG was separated by t.l.c. in the solvent system chloroform/acetone (24:1, v/v). Data are means ± S.E.M. from two experiments: N.D., not determined.

	[^{3}H]1,2-DAG (% of control)	
	30 s	300 s
Control (1 μM $PGF_{2\alpha}$)	100	100
+ Ethanol		
(0.2%)	N.D.	78.8 ± 22.0
(0.5%)	N.D.	78.3 ± 0.86*
(1%)	85.0 ± 9.17	74.2 ± 2.40*
+ Propranolol		
(10 μM)	N.D.	71.3 ± 24.0
(50 μM)	N.D.	70.3 ± 2.70*
(200 μM)	100 ± 5.60	64.3 ± 2.40*
+ U73122		
(2 μM)	38.1 ± 12.2*	N.D.
No $PGF_{2\alpha}$	28.4	23.0

* Significantly different from non-treated cells ($P < 0.05$).

agents which inhibit PLD activity on 1,2-DAG production. Upon stimulation with $PGF_{2\alpha}$ in the presence of ethanol, 1,2-DAG production in the second sustained phase was suppressed to about 70% of the control (Table 4). Furthermore, 200 μM propranolol, a non-specific but selective inhibitor of PA phosphohydrolase [35,36], decreased the $PGF_{2\alpha}$-induced 1,2-DAG production in the sustained phase, but did not affect the early-phase production (Table 4). The late 1,2-DAG production was decreased to 75% and 65% of the control in the presence of ethanol (1%) and propranolol (200 μM) respectively. In contrast, the early phase of 1,2-DAG production in response to $PGF_{2\alpha}$ was markedly decreased by the PI-PLC inhibitor, but was unchanged by ethanol and propranolol.

DISCUSSION

In the present study, we demonstrated for the first time the $PGF_{2\alpha}$-induced activation of PLD (Figure 1). The initial rapid rise (first 1–2 min) in metabolically stable [^{3}H]PEt was followed by the sustained elevation in $PGF_{2\alpha}$-stimulated cells. The same concentration of $PGF_{2\alpha}$ also caused a rapid and transient increase of $[Ca^{2+}]_i$ due to $Ins P_3$ produced by PI-PLC activation. These results suggested the relationship between two $PGF_{2\alpha}$-activated signal pathways, PI-PLC/Ca^{2+} and PLD, as described in several reports [7,8,10,16,17,19,22,34]. Pretreatment of the cells with the PI-PLC inhibitor U73122 decreased both the $PGF_{2\alpha}$-induced $[Ca^{2+}]_i$ increase and PLD activation. As demonstrated by other investigators [8–10,34], our data also indicated the requirement for a $[Ca^{2+}]_i$ increase in $PGF_{2\alpha}$-induced PLD activation in MC3T3-E1 cells. Chelation of extra- and intra-cellular free Ca^{2+} with EGTA and BAPTA diminished the $PGF_{2\alpha}$-induced $[Ca^{2+}]_i$ increase and [^{3}H]PEt production (Table 2). These results suggest that $PGF_{2\alpha}$-induced PLD activation requires a $[Ca^{2+}]_i$ increase due to PtdIns(4,5)P_2 hydrolysis by PI-PLC. Although the $[Ca^{2+}]_i$ increases by Ca^{2+} ionophores (ionomycin and A23187) or Ca^{2+}-ATPase blocker (thapsigargin) were greater than that by $PGF_{2\alpha}$, PLD activation was much lower than that with $PGF_{2\alpha}$ stimulation. These receptor-independent $[Ca^{2+}]_i$ increases could not mimic the $PGF_{2\alpha}$-induced PLD activation. Therefore $[Ca^{2+}]_i$ is

required, but is not sufficient by itself and other factor(s) may act to fulfil PLD activation. PKC is a likely candidate as one of the factors. The short (5 min) treatment with PMA (1 μM), a potent PKC activator, increased PLD activity in MC3T3-E1 cells (results not shown), as observed in other cells [8,13,14,16,17,23,43]. However, pretreatment with PKC inhibitors (H-7, calphostin C, K-252a and staurosporine) and PKC down-regulation by chronic PMA exposure did not prevent the PLD activation mediated by $PGF_{2\alpha}$. Thus $PGF_{2\alpha}$-induced activation of PLD may involve a PKC-independent pathway in these cells.

Since PLD activation (i) was stimulated by $PGF_{2\alpha}$ in digitonin-permeabilized cells at the physiological range (100 nM–1 μM) of Ca^{2+}, (ii) showed synergistic action of $PGF_{2\alpha}$ and GTP[S], and (iii) was inhibited by GDP, G-protein is strongly suggested to be involved in $PGF_{2\alpha}$-induced PLD activation. This G-protein was a pertussis-toxin-insensitive type. Taken together, it was indicated that PLD activation was elicited by a synergistic action of $[Ca^{2+}]_i$ and G-protein, but largely independent of PKC in $PGF_{2\alpha}$-stimulated MC3T3-E1 cells.

Next, we have examined the involvement of PLD activation in the sustained 1,2-DAG production via the PLD/PA phosphohydrolase pathway [22,35,37] in $PGF_{2\alpha}$-stimulated cells. In MC3T3-E1 cells, $PGF_{2\alpha}$-induced [^{3}H]1,2-DAG generation showed a biphasic increase: an initial rapid phase ($\sim$ 60 s) and a subsequent sustained phase. In the present study, the initial phase of 1,2-DAG formation was concurrent with the $[Ca^{2+}]_i$ increase and it was suppressed by addition of the PI-PLC inhibitor U73122, suggesting that the initial 1,2-DAG is derived from phosphoinositide hydrolysis via PLC. On the other hand, addition of ethanol or propranolol to inhibit PLD/PA phosphohydrolase decreased the sustained phase of 1,2-DAG formation. The same treatments were without effect on the initial 1,2-DAG phase. PLD activation alone cannot explain the sustained phase of $PGF_{2\alpha}$-induced 1,2-DAG formation; nearly one-third of total 1,2-DAG is produced via the PLD pathway. An alternative route for the sustained generation of 1,2-DAG has been thought to result from PC hydrolysis by PLC in several cell types [5,43]. Our preliminary study showed production of [^{3}H]phosphocholine in [^{3}H]choline-labelled MC3T3-E1 cells stimulated with $PGF_{2\alpha}$, thus suggesting an involvement of PC-PLC. Investigations are in progress to determine the differential contribution of the PLD and PC-PLC pathways in the late sustained elevation of 1,2-DAG in $PGF_{2\alpha}$-stimulated MC3T3-E1 cells.

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. We greatly appreciate Dr. John E. Bleasdale, The Upjohn Co. (Kalamazoo, MI, U.S.A.) for kindly providing U73122.

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Received 14 June 1993/7 October 1993; accepted 1 November 1993