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Protein Kinase C Activation Amplifies Prostaglandin $F_{2\alpha}$-Induced Prostaglandin E_2 Synthesis in Osteoblast-Like Cells

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Abstract In cloned osteoblast-like cells, MC3T3-E1, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) stimulated arachidonic acid (AA) release in a dose-dependent manner in the range between 1 nM and 10 μM. 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a protein kinase C (PKC) activator, which by itself had little effect on AA release, markedly amplified the release of AA stimulated by $PGF_{2\alpha}$ in a dose-dependent manner. 4 α-phorbol 12,13-didecanoate, a phorbol ester which is inactive for PKC, showed little effect on the $PGF_{2\alpha}$-induced AA release. 1-oleoyl-2-acetylglycerol (OAG), a specific activator for PKC, mimicked TPA by enhancement of the AA release induced by $PGF_{2\alpha}$. H-7, a PKC inhibitor, markedly suppressed the effect of OAG on $PGF_{2\alpha}$-induced AA release. Quinacrine, a phospholipase A_2 inhibitor, showed partial inhibitory effect on $PGF_{2\alpha}$-induced AA release, while it suppressed the amplification by OAG of $PGF_{2\alpha}$-induced AA release almost to the control level. Furthermore, TPA enhanced the AA release induced by melittin, known as a phospholipase A_2 activator. On the other hand, TPA inhibited the formation of inositol trisphosphate stimulated by $PGF_{2\alpha}$. Under the same condition, $PGF_{2\alpha}$ indeed stimulated prostaglandin E_2 (PGE_2) synthesis and TPA markedly amplified the $PGF_{2\alpha}$-induced PGE_2 synthesis as well as AA release. These results indicate that the activation of PKC amplifies $PGF_{2\alpha}$-induced both AA release and PGE_2 synthesis through the potentiation of phospholipase A_2 activity in osteoblast-like cells.

Key words: protein kinase C, prostaglandin, arachidonic acid, phospholipase A, osteoblast

Prostaglandins (PGs), which are synthesized from arachidonic acid (AA) by cellular enzymes, are important bioactive substances and modulates diverse cellular functions in ubiquitous cells (Samuelsson et al., 1978; Smith, 1989). Because the concentration of free AA is low (Irvine, 1982), it is considered that AA release is a rate-limiting step of PG biosynthesis. Two major pathways of AA release are generally accepted (Smith, 1989). One is the activation of phospholipase A_2 (PLA_2) which causes liberation of AA directly from esterified stores of phospholipids, and the other is the sequential phosphoinositide (PI) hydrolysis by phospholipase C (PLC) and glycerol lipases. The activities of both PLA_2 and PLC are considered to depend on intracellular Ca^{2+} level (Nozawa et al., 1991; Meldrum et al., 1991). Although precise regulatory mechanism of AA release remains unclear, evidence is accumulating that activation of protein kinase C (PKC), which is considered to be activated physiologically by diacylglycerol resulting from PI hydrolysis (Nishizuka, 1984), can modulate AA release in several kinds of cells. It has been reported that activation of PKC enhances AA release stimulated by exogenous stimuli in Madin-Darby canine kidney cells (Slivka and Insel, 1988), human platelets (Halenda et al., 1989), rabbit platelets (Murayama et al., 1990), mouse peritoneal macrophages (Balsinde et al., 1990), and rat basophilic leukemia cells (Zor et al., 1990). While, opposite results showing that PKC activation suppresses the AA release have been obtained in rabbit neutrophils (Tao et al., 1989) and rabbit platelets (Murayama et al., 1990).

In osteoblasts, as well as in other kinds of cells, PGs are considered to act as local modulators (Nijweide, 1986). Among them, prostaglandin E_2 (PGE_2) is known as a potent stimulator of bone resorption (Raisz and Martin, 1984). It has

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been reported that PGE_2 is a major eicosanoid product in osteoblasts (Raisz and Martin, 1984; Yokota, et al., 1986) including MC3T3-E1 cells derived from newborn mouse calvaria (Kodama et al., 1981; Sudo, et al., 1983) and that PGE_2 synthesis is induced by several agents such as epidermal growth factor (Yokota et al., 1986), tumor necrosis factor (Sato et al., 1987), transforming growth factors (Tashijian et al., 1985), and platelet-derived growth factor (Shupnik et al., 1982).

On the other hand, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a less potent stimulator of bone resorption than PGE_2 (Raisz and Martin, 1984; Raisz et al., 1990), has been shown to stimulate proliferation and inhibit differentiation of osteoblasts (Nijweide et al., 1986; Hakeda et al., 1987; Koshihara et al., 1989). It has been reported that $PGF_{2\alpha}$ induces PI hydrolysis in osteoblast-like MC3T3-E1 cells (Hakeda et al., 1987). We have also reported that PI hydrolysis induced by $PGF_{2\alpha}$ is pertussis toxin-sensitive event in these cells (Miwa et al., 1990). As inositol trisphosphate (IP_3) resulting from PI hydrolysis is well known to mobilize intracellular Ca^{2+} (Berridge and Irvine, 1984), by which PLA_2 could be activated (Nozawa et al., 1991), it is possible that AA release is induced by $PGF_{2\alpha}$ through the activation of PLA_2 or subsequential PI hydrolysis. Indeed, recent report has shown that $PGF_{2\alpha}$ stimulates AA release and PGE_2 production in cultured neonatal mouse calvariae (Raisz et al., 1990). However, the precise mechanism of AA release induced by $PGF_{2\alpha}$ and the effect of PKC activation on the AA release has not been elucidated yet.

The present study was designed to clarify the effect of PKC activation on $PGF_{2\alpha}$-induced AA release in osteoblast-like MC3T3-E1 cells. Herein, we show that the activation of PKC amplifies $PGF_{2\alpha}$-induced both AA release and PGE_2 synthesis through the potentiation of PLA_2 activity in osteoblast-like cells.

METHODS

Materials

[5,6,8,9,11,12,14,15-^{3}H(N)]AA (76.0 Ci/mmol) was purchased from Du Pont/NEN. *Myo*-[2-^{3}H]inositol (81.5 Ci/mmol) and PGE_2 [^{125}I] assay system were purchased from Amersham International. $PGF_{2\alpha}$, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 4α-phorbol 12,13-didecanoate (4α-PDD), quinacrine, melittin, and essentially fatty acid-free bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, MO. 1-oleoyl-2-acetylglycerol (OAG) was purchased from Nacalai Tesque, Inc., Kyoto, Japan. 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) and N-(2-guanidineethyl)-5-isoquinoline-sulfonamide hydrochloride (HA1004) were purchased from Seikagaku Kogyo, Tokyo, Japan. Other materials and chemicals were obtained from commercial sources.

Cell Culture

MC3T3-E1 cells were generously provided by Dr. M. Kumegawa (Meikai University, Sakado, Japan) and maintained in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells (5×10^4) were seeded into 35 mm diameter dishes in 2 ml of α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. For the experiment of the formation of IP_3, the medium was exchanged for inositol-free α-MEM containing 0.3% FCS. The cells were used for experiments 48 h thereafter.

Measurement of AA Release

The cultured cells were labeled with [^{3}H]AA (0.3 μCi/dish) for 24 h. Then the medium was removed and the cells were washed with 1 ml of buffer A [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, containing 135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, and 1 mM $CaCl_2$] four times. The cells were preincubated subsequently with 1 ml of buffer A containing 0.1% essentially fatty acid-free BSA at 37°C for 20 min, and then the cells were stimulated by $PGF_{2\alpha}$ or melittin. The reaction was terminated by collecting the medium and the radioactivity of the medium was measured. When indicated, the cells were treated with TPA, 4α-PDD, OAG, H-7, HA1004, or quinacrine for 20 min prior to the stimulation.

Measurement of the Formation of IP_3

The cultured cells were labeled with *myo*-[^{3}H]inositol (3 μCi/dish) for 48 h. The labeled cells were washed twice with 1 ml of buffer B (5 mM HEPES, pH 7.4, containing 150 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.8 mM $MgSO_4$, and 1 mM $CaCl_2$) and preincubated with various doses of TPA in 1 ml of buffer B containing 0.01% BSA at 37°C for 20 min. For the last 10 min, the cells were pretreated with 10 mM LiCl.

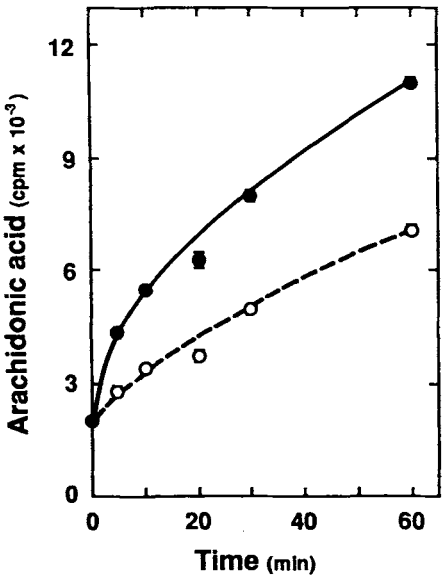


Fig. 1. Effect of $PGF_{2\alpha}$ on AA release in MC3T3-E1 cells. The [^{3}H]AA-labeled cells were stimulated by 10 μM $PGF_{2\alpha}$ (●) or control (○) for indicated periods, and then the release of AA was determined. Each value represents the mean ± S.D. of triplicate determinations.

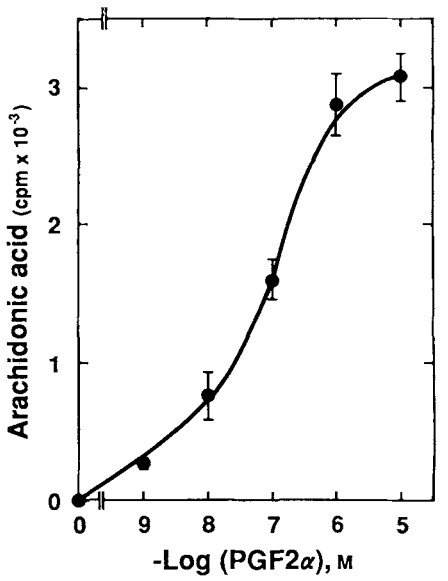


Fig. 2. Dose-dependent stimulation by $PGF_{2\alpha}$ of AA release in MC3T3-E1 cells. The [^{3}H]AA-labeled cells were stimulated by various doses of $PGF_{2\alpha}$ for 30 min. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± S.D. of triplicate determinations.

After the pretreatment, the cells were stimulated by 10 μM $PGF_{2\alpha}$ for 30 min. The reaction was terminated by 15% trichloroacetic acid. The acid supernatant was treated with diethyl ether to remove the acid and then neutralized with NaOH. The supernatant was applied to a column of Dowex AG1-X8 formate form. To remove inositol monophosphate and inositol bisphosphate, 8 ml of 0.1 M formic acid containing 0.4 M ammonium formate was applied to the column. Then the radioactive IP_3 were eluted from the column with 8 ml of 0.1 M formic acid containing 1 M ammonium formate (Berridge et al., 1983, 1984).

Measurement of PGE_2 Synthesis

Procedures were done as described under "Measurement of AA Release" except for using unlabeled cells. PGE_2 in the medium was determined with a radioimmunoassay kit.

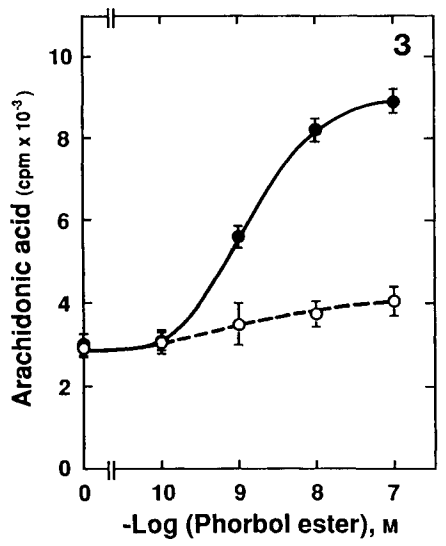
Statistical Analysis

The data were analyzed by Student's t test.

RESULTS

$PGF_{2\alpha}$ (10 μM) significantly stimulated the AA release in osteoblast-like MC3T3-E1 cells (Fig. 1). The AA release induced by $PGF_{2\alpha}$ was dose dependent in the range between 1 nM and 10 μM (Fig. 2). Pretreatment with TPA, a PKC activating phorbol ester (Nishizuka, 1984), which by itself had little effect on AA release in these cells (data not shown), markedly amplified the 10 μM $PGF_{2\alpha}$-induced AA release in a dose-dependent manner in the range between 0.1 nM and 0.1 μM (Fig. 3). TPA at 0.1 μM caused about 3-fold enhancement of 10 μM $PGF_{2\alpha}$-induced AA release. 4 α-PDD, a phorbol ester which is known to be inactive for PKC (Nishizuka, 1984), had little effect on the AA release (Fig. 3). OAG, a synthetic diacylglycerol known to be a specific activator of PKC (Nishizuka, 1984), which alone had little effect on AA release as well as TPA (data not shown), also amplified the AA release induced by 10 μM $PGF_{2\alpha}$ dose-dependently between 1 μM and 0.1 mM (Fig. 4). To confirm the involvement of PKC in the enhancement of $PGF_{2\alpha}$-induced AA release by OAG, we examined the effect of H-7, a PKC inhibitor (Hidaka et al., 1984), on the enhancement by OAG. H-7 (20 μM) suppressed the OAG effect almost to the control level (Fig. 5). HA1004, a control for H-7 (Hidaka et al., 1984), showed little effect on the OAG effect (Fig. 5).

To elucidate whether PLA_2 is involved in the $PGF_{2\alpha}$-induced AA release and its enhancement by OAG, we tested the effects of quinacrine, a PLA_2 inhibitor (Lapetina et al., 1981), on these



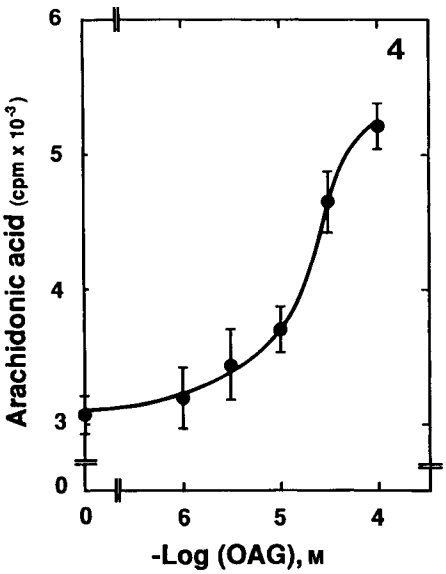


Fig. 3. Effects of phorbol esters on $PGF_{2\alpha}$-induced AA release in MC3T3-E1 cells. The [^{3}H]AA-labeled cells were pretreated with various doses of TPA (●) or 4α-PDD (○) for 20 min, and then stimulated by 10 μM $PGF_{2\alpha}$ for 30 min. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± S.D. of triplicate determinations.

Fig. 4. Effect of OAG on $PGF_{2\alpha}$-induced AA release in MC3T3-E1 cells. The [^{3}H]AA-labeled cells were pretreated with various doses of OAG for 20 min, and then stimulated by 10 μM $PGF_{2\alpha}$ for 30 min. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± S.D. of triplicate determinations.

Fig. 5. Effect of H-7 on amplification of $PGF_{2\alpha}$-induced AA release by OAG in MC3T3-E1 cells. The [^{3}H]AA-labeled cells were pretreated with 20 μM H-7 or 20 μM HA1004 in the presence of 0.1 mM OAG for 20 min, and then stimulated by 10 μM $PGF_{2\alpha}$ for 30 min. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± S.D. of triplicate determinations. $^{**}P < 0.01$, as compared to the value treated with HA1004.

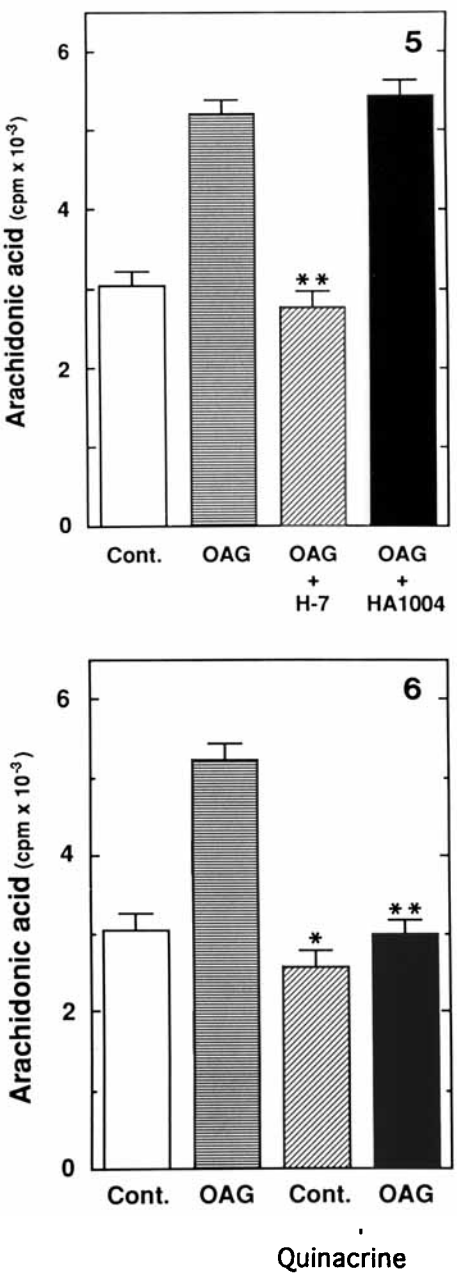


Fig. 6. Effect of quinacrine on $PGF_{2\alpha}$-induced AA release and its amplification by OAG in MC3T3-E1 cells. The [^{3}H]AA-labeled cells were pretreated with 10 μM quinacrine in the presence of 0.1 mM OAG or vehicle for 20 min, and then stimulated by 10 μM $PGF_{2\alpha}$ for 30 min. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± S.D. of triplicate determinations. $^{*}P < 0.05$, as compared to the control; $^{**}P < 0.01$, as compared to the value treated with OAG alone.

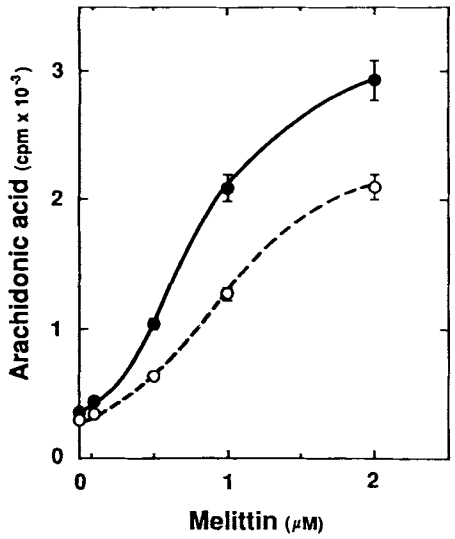


Fig. 7. Effect of TPA on melittin-induced AA release in MC3T3-E1 cells. The [^{3}H]AA-labeled cells were pretreated with 0.1 μM TPA (●) or vehicle (○) for 20 min, and then stimulated by various doses of melittin for 30 min. Each value represents the mean ± S.D. of triplicate determinations.

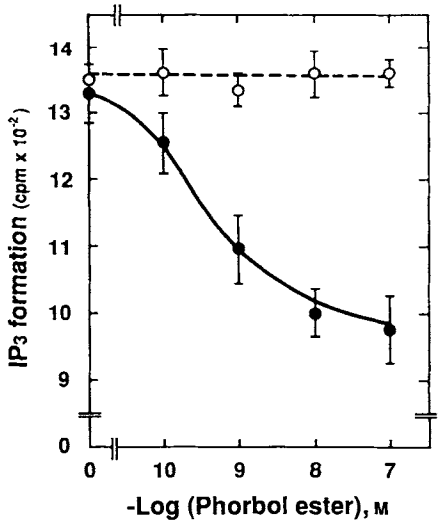


Fig. 8. Effects of phorbol esters on $PGF_{2\alpha}$-induced IP_3 formation in MC3T3-E1 cells. The [^{3}H]inositol-labeled cells were pretreated with various doses of TPA (●) or 4α-PDD (○) for 20 min, and then stimulated by 10 μM $PGF_{2\alpha}$ for 30 min. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± S.D. of triplicate determinations.

reactions. Treatment with 10 μM quinacrine resulted in about 16% suppression of 10 μM $PGF_{2\alpha}$-induced AA release (Fig. 6). While the OAG-induced enhancement of AA release stimulated by 10 μM $PGF_{2\alpha}$ was inhibited by 10 μM quinacrine almost to the control level (Fig. 6). Furthermore, we examined the effect of TPA on the AA release induced by melittin, a PLA_2 activator (Mollay and Kreil, 1974; Shier, 1979). Melittin stimulated AA release potently in a dose-dependent manner between 0.1 μM and 2 μM in these cells (Fig. 7). TPA (0.1 μM) also enhanced melittin-induced AA release (Fig. 7).

We next examined the effects of phorbol esters on $PGF_{2\alpha}$-induced PI hydrolysis. As previously described (Miwa et al., 1990), 10 μM $PGF_{2\alpha}$ for 30 min significantly stimulated the formation of IP_3 (Fig. 8). Pretreatment with TPA, which by itself had no effect on the formation of IP_3 (data not shown), suppressed the 10 μM $PGF_{2\alpha}$-induced IP_3 formation dose-dependently between 0.1 nM and 0.1 μM. TPA at 0.1 μM resulted in about 27% suppression of 10 μM $PGF_{2\alpha}$-induced IP_3 formation. However, 4α-PDD was ineffective in this capacity (Fig. 8).

In addition, we investigated that the effect of $PGF_{2\alpha}$ on the synthesis of PGE_2 and the effect of TPA on the $PGF_{2\alpha}$-induced PGE_2 synthesis. Ten μM $PGF_{2\alpha}$ markedly stimulated PGE_2 synthesis and 0.1 μM TPA potently amplified the synthesis of PGE_2 stimulated by $PGF_{2\alpha}$ (Table I). As well as observed in [^{3}H]AA release, TPA enhanced $PGF_{2\alpha}$-induced PGE_2 synthesis.

TABLE I. Effects of TPA on $PGF_{2\alpha}$-Induced PGE_2 Synthesis in MC3T3-E1 Cells*

	PGE_2 synthesis (pg/dish)
Control	25 ± 14
10 μM $PGF_{2\alpha}$	1,200 ± 141[a]
Control + 0.1 μM TPA	134 ± 22[b]
10 μM $PGF_{2\alpha}$ + 0.1 μM TPA	4,317 ± 494[c]

*The cultured cells were pretreated with 0.1 μM TPA for 20 min, then stimulated by 10 μM $PGF_{2\alpha}$ or control for 90 min. Each value represents the mean ± S.D. of triplicate determinations.
[a]$P < 0.01$.
[b]$P < 0.05$, as compared to the control.
[c]$P < 0.01$, as compared to the value stimulated by $PGF_{2\alpha}$ without TPA pretreatment.

DISCUSSION

In the present study, we showed that TPA markedly amplified the $PGF_{2\alpha}$-induced AA release, but 4α-PDD was inactive in this capacity in osteoblast-like MC3T3-E1 cells. It is well known that TPA, but not 4α-PDD, activates PKC (Nishizuka, 1984). Hence, the effect of TPA on $PGF_{2\alpha}$-induced AA release seems to be

mediated through the activation of PKC. The fact that OAG, a specific PKC activator, mimicked TPA by amplifying of $PGF_{2\alpha}$-induced AA release also supports this notion. Furthermore, we demonstrated that H-7, a PKC inhibitor (Hidaka et al., 1984), abolished the effect of OAG on $PGF_{2\alpha}$-induced AA release almost completely compared with HA1004, a control for H-7 (Hidaka et al., 1984). Thus, these results strongly suggest that the activation of PKC potently amplifies the AA release induced by $PGF_{2\alpha}$ in osteoblast-like cells.

To clarify the mechanism of amplification by PKC, we examined the effect of quinacrine, a PLA_2 inhibitor (Lapetina et al., 1981), on $PGF_{2\alpha}$-induced AA release in PKC-activated or nonactivated MC3T3-E1 cells. Quinacrine inhibited the AA release induced by $PGF_{2\alpha}$ slightly in PKC-nonactivated cells; however, it abolished the amplification by PKC in $PGF_{2\alpha}$-induced AA release almost to the control level. Moreover, we examined the effect of PKC activation on melittin-, a PLA_2 activator (Mollay and Kreil, 1974; Shier, 1979), induced AA release. TPA markedly enhanced the AA release induced by melittin. Since neither TPA nor OAG had significant effect on AA release by itself, it seems unlikely that PKC activates PLA_2 directly. Namely, phospholylation of some kind(s) of substance(s) by PKC seems to alter the sensitivity of PLA_2 activity to $PGF_{2\alpha}$ stimulation. These results strongly suggest that the modification of PLA_2 sensitivity induced by PKC activation plays an important role in PKC-induced amplification of $PGF_{2\alpha}$-induced AA release. As previously described, we have shown that $PGF_{2\alpha}$ stimulates PI hydrolysis in these cells (Miwa et al., 1990). Thus, it is considered that Ca^{2+} mobilization is occurred through PI hydrolysis induced by $PGF_{2\alpha}$. Recently, evidence is accumulating that PKC acts to modulate the sensitivity of PLA_2 to Ca^{2+} (Carter et al., 1989; Chardonnens et al., 1990). So, in osteoblast-like MC3T3-E1 cells, it is possible that similar mechanism may be involved in the amplification of $PGF_{2\alpha}$-induced AA release by PKC.

We also showed that TPA inhibited the $PGF_{2\alpha}$-induced formation of IP_3 but 4α-PDD was ineffective in these cells. These results suggest that the activation of PKC inhibits PI hydrolysis induced by $PGF_{2\alpha}$. Therefore, it seems unlikely that AA release from sequential hydrolysis of PI by PLC and glycerol lipases is a major pathway of PKC-induced amplification of the AA release in osteoblast-like cells.

Furthermore, we showed that $PGF_{2\alpha}$ stimulated PGE_2 synthesis and that TPA enhanced the $PGF_{2\alpha}$-induced PGE_2 synthesis potently as well as AA release. TPA by itself had subtle but marked effect on PGE_2 synthesis; however, enhancement of $PGF_{2\alpha}$-induced PGE_2 synthesis was more significant. As $PGF_{2\alpha}$ has been reported to stimulate bone resorption but to be less potent than PGE_2 (Raisz and Martin, 1984; 1990) and as $PGF_{2\alpha}$ stimulates PI hydrolysis resulting in PKC activation in these cells (Hakeda et al., 1987; Miwa et al., 1990), these results suggest that there is a positive feedback mechanism in $PGF_{2\alpha}$-induced modulation of osteoblast functions in which enhancement of PGE_2 synthesis through PKC activation induced by $PGF_{2\alpha}$ itself plays an important role.

In a previous report, we have shown that PKC is involved in the process which suppresses differentiation of osteoblast-like MC3T3-E1 cells and directs them toward proliferation (Kozawa et al., 1989). Additionally, in this study, we demonstrated that PKC potentiated PLA_2 sensitivity resulting in enhancement of capacity of PGE_2 synthesis, a potent stimulator of bone resorption (Raisz and Martin, 1984). These results can lead us to speculate that PKC enhances bone resorption in metabolism of bone. Further investigation would be required to clarify details.

In conclusion, our results indicate that the activation of PKC amplifies $PGF_{2\alpha}$-induced both AA release and PGE_2 synthesis through the potentiation of PLA_2 activity in osteoblast-like cells.

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