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Prostaglandin $F_{2\alpha}$ Activates Phospholipase D Independently From Activation of Protein Kinase C in Osteoblast-Like Cells

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Abstract We previously reported that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptor is coupled to pertussis toxin (PTX)–sensitive GTP-binding protein (G protein) in osteoblast-like MC3T3-E1 cells [Miwa et al. (1990): Biochem Biophys Res Commun 171:1229–1235]. In the present study, we examined the effect of $PGF_{2\alpha}$ on the activation of phosphatidylcholine-hydrolyzing phospholipase D in MC3T3-E1 cells. $PGF_{2\alpha}$ stimulated the formation of choline in a dose-dependent manner in the range between 10 nM and 10 μM. The formation of choline was stimulated by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a protein kinase C (PKC)–activating phorbol ester. 4α-Phorbol 12,13-didecanoate, a PKC-nonactivating phorbol ester, had little effect on choline formation. The formation of choline stimulated by a combination of $PGF_{2\alpha}$ and TPA was additive. Staurosporine, an inhibitor for protein kinases, which inhibited the effect of TPA on choline formation, dose-dependently enhanced the formation of choline induced by $PGF_{2\alpha}$. NaF, an activator of G protein, stimulated the formation of choline. The formation of choline stimulated by a combination of $PGF_{2\alpha}$ and NaF was not additive. NaF-induced formation of choline was dose-dependently enhanced by staurosporine. PTX dose-dependently inhibited the $PGF_{2\alpha}$-induced formation of choline. These results strongly suggest that $PGF_{2\alpha}$ activates phospholipase D independently from the activation of PKC in osteoblast-like cells and PTX-sensitive G protein is involved in the $PGF_{2\alpha}$-induced phospholipase D activation.

Key words: prostaglandin $F_{2\alpha}$, phospholipase D, protein kinase C, pertussis toxin, GTP-binding protein, osteoblast

It is generally accepted that, in response to a variety of agonists, phosphoinositides are hydrolyzed by phospholipase C, resulting in the formation of diacylglycerol and inositol phosphates [Berridge and Irvine, 1984; Nishizuka, 1986]. Among these products, diacylglycerol serves as a second messenger for the activation of protein kinase C (PKC). In a previous study [Kozawa et al., 1989], we have shown that, in osteoblast-like MC3T3-E1 cells cloned from newborn mouse calvaria [Kodama et al., 1981; Sudo et al., 1983], the activation of PKC is involved in the process which suppresses the differentiation of these cells and directs them toward proliferation.

Phosphoinositide hydrolysis is not the only pathway of diacylglycerol formation [Exton, 1990; Billah and Anthes, 1990]. It is well known that phospholipase D catalyzes the hydrolysis of phosphatidylcholine, resulting in the formation of phosphatidic acid, which is a precursor of diacylglycerol [Exton, 1990]. Although it has been shown that phosphatidylcholine hydrolysis by phospholipase D is secondary to the activation of PKC, recent evidence suggests that phospholipase D is also activated via an independent pathway from PKC activation [Exton, 1990; Billah and Anthes, 1990]. It is nowadays recognized that phospholipase D plays an important role in modulating cellular functions that require long-term activation of PKC, since phosphatidylcholine is the principal phospholipid in cell membranes [Exton, 1990; Billah and Anthes, 1990]. However, the precise regulatory mechanism of phospholipase D activation in osteoblasts has not yet been clarified.

Prostaglandins are recognized to act as local modulators in osteoblasts [Nijweide et al., 1986].

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Among them, it has been reported that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) stimulates the proliferation and inhibits the differentiation of osteoblasts [Nijweide et al., 1986; Hakeda et al., 1987; Koshihara and Kawamura, 1989]. In a previous study [Miwa et al., 1990], we have shown that $PGF_{2\alpha}$ stimulates phosphoinositide hydrolysis via pertussis toxin–sensitive GTP-binding protein in osteoblast-like MC3T3-E1 cells. In the present study, we examined the effect of $PGF_{2\alpha}$ on phosphatidylcholine-hydrolyzing phospholipase D activity in MC3T3-E1 cells. Herein we show that $PGF_{2\alpha}$ activates phospholipase D independently from the activation of PKC in osteoblast-like cells and pertussis toxin–sensitive GTP-binding protein is involved in the $PGF_{2\alpha}$-induced phospholipase D activation.

METHODS

Materials

[methyl-^{3}H]Choline chloride (85 Ci/mmol) was purchased from Amersham Japan (Tokyo, Japan). $PGF_{2\alpha}$, NaF, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 4α-phorbol 12,13-didecanoate (4α-PDD), and staurosporine were purchased from Sigma Chemical Co. (St. Louis, MO). Pertussis toxin was purchased from Funakoshi Pharmaceutical Co. (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources.

Cell Culture

Cloned osteoblast-like 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 2 ml of α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h. When indicated, the cells were pretreated with various doses of pertussis toxin for 24 h.

Measurement of the Formation of Choline

To determine phospholipase D activity in osteoblast-like MC3T3-E1 cells, the cultured cells were labeled with [methyl-^{3}H]choline chloride (2 μCi/dish) for 24 h. The labeled cells were washed twice with 1 ml of an assay buffer (5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 150 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.8 mM $MgSO_4$, and 1 mM $CaCl_2$) and subsequently incubated in 1 ml of the assay buffer containing 0.01% bovine serum albumin (BSA) at 37°C for 20 min. The cells were then stimulated by $PGF_{2\alpha}$, TPA, 4α-PDD, or NaF. The reaction was terminated by adding 0.75 ml of ice-cold methanol. The dishes stood on ice for 10 min. The contents were transferred to tubes to which chloroform was added and stood on ice for 60 min. Chloroform and water were then added to a final ratio of 1:1:0.9 (chloroform: methanol:water). The tubes were centrifuged at 14,000*g* for 5 min, and the upper aqueous methanolic phase was taken for analysis of the water-soluble choline-containing metabolites. The methanolic phase was separated on a 1 ml of Dowex 50-WH^+ column as described [Cook and Wakelam, 1989] with a minor modification. In brief, the phase was diluted to 5 ml with water and applied to the column. Glycerophosphocholine and choline phosphate were removed with 24 ml of water, and choline was eluted with 8 ml of 1 M HCl. When indicated, the cells were pretreated with staurosporine for 20 min.

Determination

The radioactivity of ^{3}H samples was determined with a Beckman LS-6000IC liquid scintillation spectrometer.

Statistical Analysis

The data were analyzed by Student's *t*-test and $P < 0.05$ was considered significant. All data are presented as the mean ± SEM of triplicate determinations.

RESULTS

Effect of $PGF_{2\alpha}$ on the Formation of Choline in MC3T3-E1 Cells

$PGF_{2\alpha}$ significantly stimulated the formation of choline in osteoblast-like MC3T3-E1 cells (Fig. 1). The formation of choline stimulated by $PGF_{2\alpha}$ increased up to 20 min in these cells. The effect of $PGF_{2\alpha}$ was dose-dependent in the range between 10 nM and 10 μM (Fig. 2). The maximum effect of $PGF_{2\alpha}$ was observed at 3 μM.

Effect of Phorbol Esters on the Formation of Choline in MC3T3-E1 Cells

TPA, which is known to be a PKC-activating phorbol ester [Nishizuka, 1986], markedly stimu-

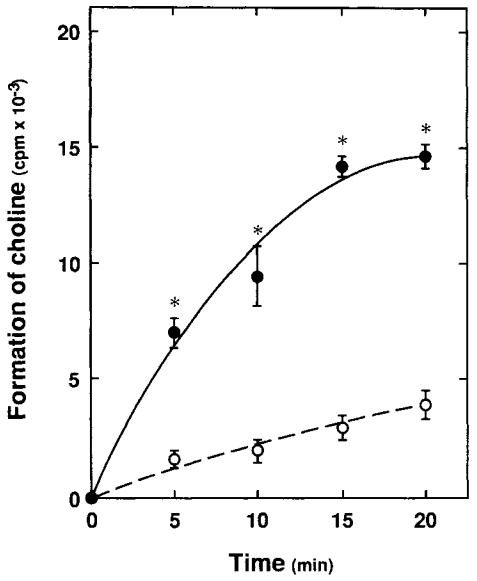

Fig. 1. Effect of $PGF_{2\alpha}$ on the formation of choline in MC3T3-E1 cells. The labeled cells were stimulated by 3 μM $PGF_{2\alpha}$ (●) or vehicle (○) for the indicated periods. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *, $P < 0.05$ compared to the control value.

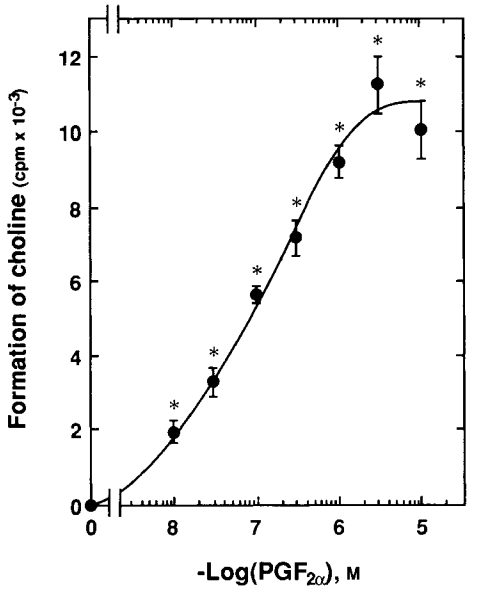

Fig. 2. Dose-dependent effect of $PGF_{2\alpha}$ on the formation of choline in MC3T3-E1 cells. The labeled cells were stimulated by various doses of $PGF_{2\alpha}$ for 15 min. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *, $P < 0.05$ compared to the control value.

lated the formation of choline in MC3T3-E1 cells. The formation of choline induced by TPA increased up to 20 min in these cells (Fig. 3A). The effect of TPA was dose-dependent in the range between 0.1 nM and 0.1 μM (Fig. 3B). The maximum effect of TPA was observed at 0.1 μM. In contrast, 4α-PDD (0.1 μM), which is known to be ineffective in PKC activation [Nishizuka, 1986], had little effect on the formation of choline (Fig. 3A). When the cells were stimulated by a combination of a maximum dose of $PGF_{2\alpha}$ (3 μM) and that of TPA (0.1 μM), the effect on the formation of choline was additive (12,073 ± 468 cpm for 3 μM $PGF_{2\alpha}$ alone; 51,270 ± 2,868 cpm for 0.1 μM TPA alone; 65,515 ± 3,012 cpm for a combination of 3 μM $PGF_{2\alpha}$ and 0.1 μM TPA; values for unstimulated cells have been subtracted from each data point as measured during a stimulation for 15 min).

Effects of Staurosporine on the Formation of Choline Induced by TPA and $PGF_{2\alpha}$ in MC3T3-E1 Cells

To clarify whether PKC activation is involved in $PGF_{2\alpha}$-induced formation of choline in MC3T3-E1 cells, we examined the effect of staurosporine, an inhibitor for protein kinases [Tamaoki et al., 1986], on the formation of choline induced by $PGF_{2\alpha}$. Staurosporine, which by itself had little effect on choline formation (data not shown), inhibited the formation of choline induced by a maximum dose of TPA (0.1 μM) dose-dependently in the range between 0.1 and 3 μM in these cells (Fig. 4A). On the other hand, the $PGF_{2\alpha}$-induced formation of choline was significantly enhanced by staurosporine in a dose-dependent manner in the range between 0.1 and 3 μM (Fig. 4B).

Effect of NaF on the Formation of Choline in MC3T3-E1 Cells

NaF, a nonspecific activator of GTP-binding protein [Gilman, 1986], stimulated the formation of choline in a dose-dependent manner in the range between 5 and 40 mM in MC3T3-E1 cells (data not shown). The maximum effect of NaF was observed at 40 mM. The formation of choline stimulated by a combination of $PGF_{2\alpha}$ (3 μM) and NaF (40 mM) was not additive (Fig. 5). Staurosporine significantly enhanced the formation of choline induced by NaF in a dose-dependent manner in the range between 0.1 and 3 μM as well as that by $PGF_{2\alpha}$ (Fig. 6).

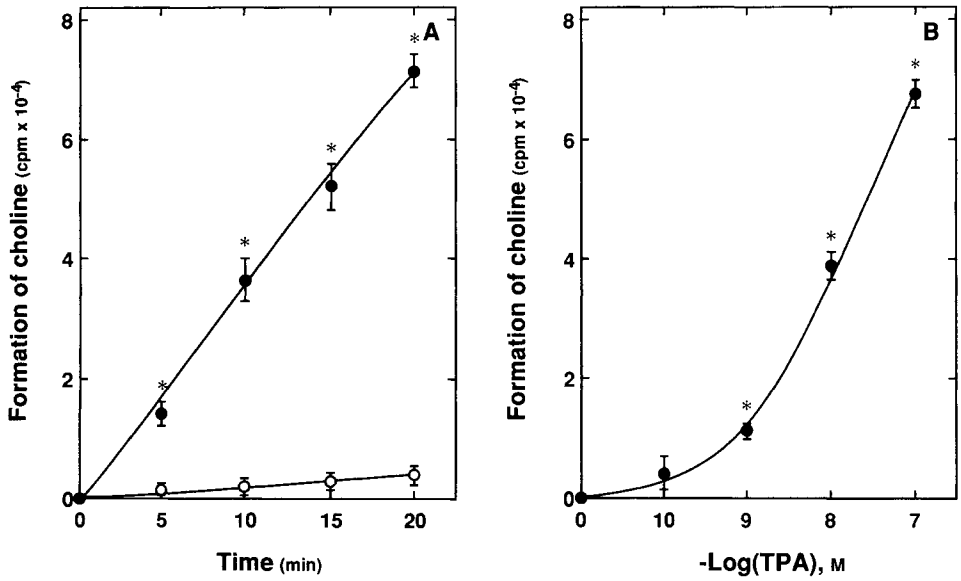

Fig. 3. Effects of phorbol esters on the formation of choline in MC3T3-E1 cells. **A:** The labeled cells were stimulated by 0.1 μM TPA (●) or 0.1 μM 4α-PDD (○) for the indicated periods. *, $P < 0.05$ compared to the value stimulated by 4α-PDD. **B:** The labeled cells were stimulated by various doses of TPA for 20 min. *, $P < 0.05$ compared to the control value. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

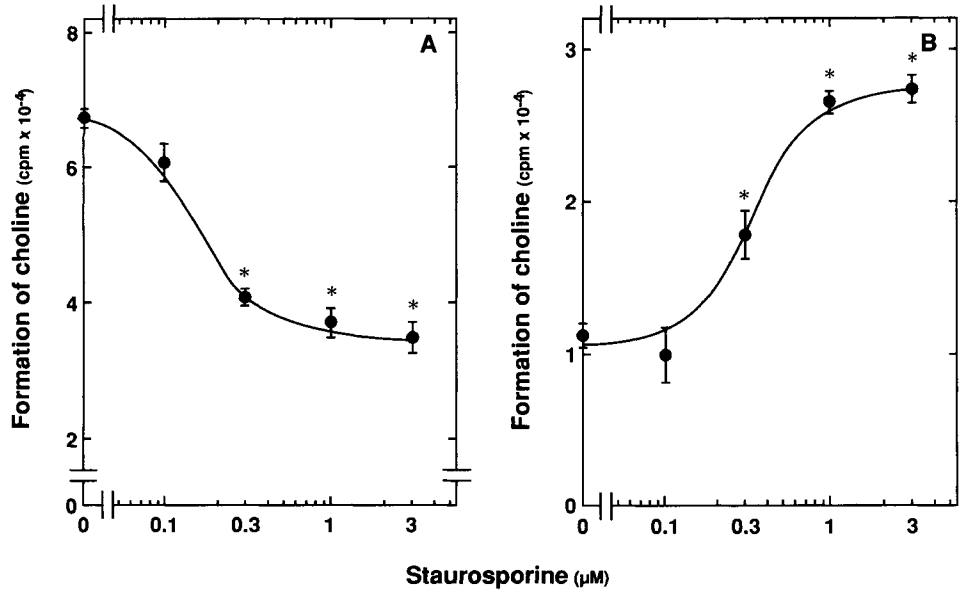

Fig. 4. Effects of staurosporine on TPA- or $PGF_{2\alpha}$-induced formation of choline in MC3T3-E1 cells. The labeled cells were pretreated with various doses of staurosporine for 20 min and *then stimulated by* 0.1 μM TPA (**A**) or 3 μM PGF_2 (**B**) for 20 min. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *, $P < 0.05$ compared to the value without staurosporine pretreatment.

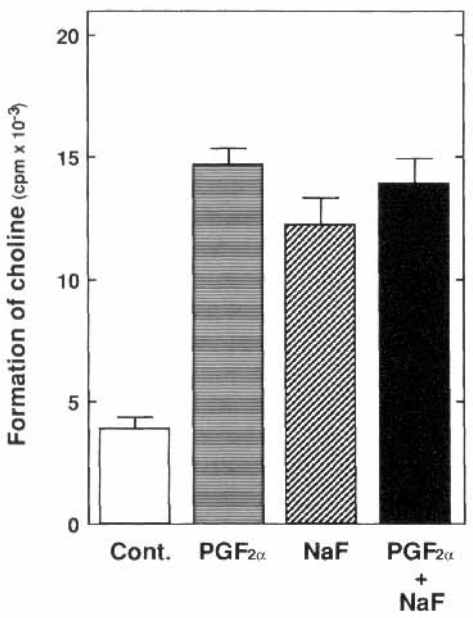

Fig. 5. Effect of NaF on $PGF_{2\alpha}$-induced formation of choline in MC3T3-E1 cells. The labeled cells were stimulated by 3 μM $PGF_{2\alpha}$ or 40 mM NaF for 20 min. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

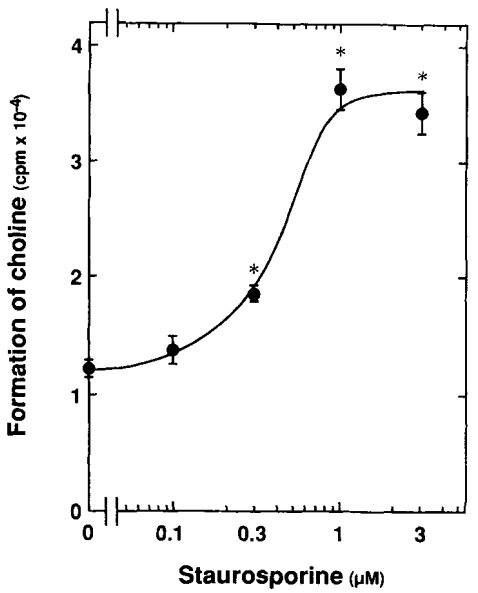

Fig. 6. Effect of staurosporine on NaF-induced formation of choline in MC3T3-E1 cells. The labeled cells were pretreated with various doses of staurosporine for 20 min and then stimulated by 40 mM NaF for 20 min. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *, $P < 0.05$ compared to the value without staurosporine pretreatment.

Effect of Pertussis Toxin on the Formation of Choline Induced by $PGF_{2\alpha}$ in MC3T3-E1 Cells

In a previous study [Miwa et al., 1990], we have reported that $PGF_{2\alpha}$ receptor is coupled to pertussis toxin–sensitive GTP-binding protein in MC3T3-E1 cells. We next examined the effect of pertussis toxin on $PGF_{2\alpha}$-induced formation of choline in these cells. The pretreatment with pertussis toxin partially but significantly inhibited the formation of choline induced by $PGF_{2\alpha}$ in a dose-dependent manner in the range between 1 ng/ml and 1 μg/ml (Fig. 7). The maximum inhibitory effect of pertussis toxin (1 μg/ml) on the formation of choline induced by 3 μM $PGF_{2\alpha}$ was 38%. We previously demonstrated that the membrane from MC3T3-E1 cells pretreated with 1 μg/ml pertussis toxin cannot be further ADP-ribosylated [Tokuda et al., 1991]. Thus, this partial inhibition does not seem to be due to an incomplete ADP-ribosylation.

DISCUSSION

In the present study, we showed that $PGF_{2\alpha}$ and TPA stimulated the formation of choline in osteoblast-like MC3T3-E1 cells. In addition, we demonstrated that 4α-PDD had little effect on the choline formation, and staurosporine, an inhibitor for protein kinases [Tamaoki et al., 1986], inhibited the TPA-induced formation of choline in these cells. It is well known that TPA is a PKC activator, but 4α-PDD is ineffective for PKC activation [Nishizuka, 1986]. Therefore, our findings suggest that the effect of TPA on the formation of choline is mediated through the activation of PKC in MC3T3-E1 cells. In this study, we showed that the effect of TPA on the choline formation was more potent than that of $PGF_{2\alpha}$. It is well recognized that TPA is hardly degraded [Nishizuka, 1986]. Therefore, TPA may extend the choline formation more than a physiologically limited response in MC3T3-E1 cells.

In a previous study [Miwa et al., 1990], we have reported that $PGF_{2\alpha}$ significantly stimulates phosphoinositide hydrolysis in MC3T3-E1 cells. It is well established that phosphoinositides are hydrolyzed by phospholipase C, resulting in the formation of diacylglycerol and inositol phosphates. Among these products, diacylglycerol serves as a messenger for the activation of PKC [Berridge and Irvine, 1984; Nishizuka, 1986]. So, it is most likely that $PGF_{2\alpha}$ activates PKC in MC3T3-E1 cells. However, in

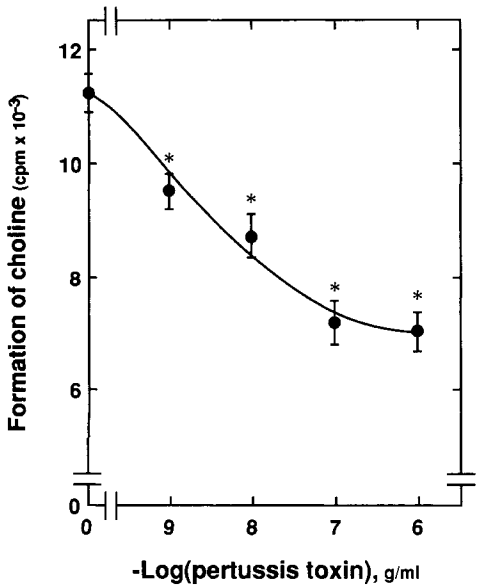

Fig. 7. Effect of pertussis toxin on the $PGF_{2\alpha}$-induced formation of choline in MC3T3-E1 cells. The labeled cells were pretreated with various doses of pertussis toxin for 24 h and then stimulated by 3 μM $PGF_{2\alpha}$ for 20 min. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *, $P < 0.05$ compared to the value without pertussis toxin pretreatment.

this study, we showed that the formation of choline by a combination of $PGF_{2\alpha}$ and TPA was additive at a maximum dose. In addition, we also demonstrated that staurosporine significantly enhanced the formation of choline induced by $PGF_{2\alpha}$ in these cells, though staurosporine suppressed the TPA-induced formation of choline. Since staurosporine is known to be an inhibitor for protein kinases [Tamaoki et al., 1986], staurosporine may enhance phospholipase D activated by $PGF_{2\alpha}$ through the inhibition of uncharacterized kinases in MC3T3-E1 cells. So, it seems unlikely that the formation of choline induced by $PGF_{2\alpha}$ is through the activation of PKC. Therefore, our findings suggest that $PGF_{2\alpha}$ stimulates phospholipase D independently from the activation of PKC in osteoblast-like MC3T3-E1 cells.

We next showed that NaF, a GTP-binding protein activator [Gilman, 1986], mimicked $PGF_{2\alpha}$ by stimulating the formation of choline in osteoblast-like MC3T3-E1 cells and that the formation of choline stimulated by a combination of $PGF_{2\alpha}$ and NaF was not additive. Furthermore, we demonstrated that staurosporine which inhibited TPA-induced formation of choline significantly enhanced the formation of choline induced by NaF as well as that by $PGF_{2\alpha}$. In addition, we demonstrated that pertussis toxin inhibited the formation of choline in these cells. We previously showed that $PGF_{2\alpha}$ receptor is coupled to pertussis toxin–sensitive GTP-binding protein in MC3T3-E1 cells [Miwa et al., 1990]. Therefore, our findings suggest that pertussis toxin–sensitive GTP-binding protein is involved in $PGF_{2\alpha}$-induced activation of phospholipase D in MC3T3-E1 cells.

It is well recognized that $PGF_{2\alpha}$ has a potent mitogenic effect on osteoblasts [Nijweide et al., 1986; Hakeda et al., 1987]. $PGF_{2\alpha}$ has been reported to stimulate proliferation of osteoblast-like MC3T3-E1 cells through phosphoinositide hydrolysis [Hakeda et al., 1987], and we have shown that pertussis toxin–sensitive GTP-binding protein is involved in $PGF_{2\alpha}$-induced phosphoinositide hydrolysis in MC3T3-E1 cells [Miwa et al., 1990]. In a previous study [Kozawa et al., 1989], we have reported that PKC is involved in the process which directs osteoblast-like MC3T3-E1 cells toward proliferation. It is generally accepted that PKC is activated by diacylglycerol [Nishizuka, 1986]. Diacylglycerol is recognized to be produced not only from phosphoinositide hydrolysis by phospholipase C, but also from phosphatidylcholine hydrolysis by phospholipase D [Nishizuka, 1986; Exton, 1990; Billah and Anthes, 1990]. Therefore, these results suggest that $PGF_{2\alpha}$ activates PKC through the stimulation of both phopholipase C and phospholipase D in osteoblast-like MC3T3-E1 cells resulting in the direction of proliferation.

In conclusion, our results strongly suggest that $PGF_{2\alpha}$ activates phospholipase D independently from the activation of PKC in osteoblast-like cells and pertussis toxin–sensitive GTP-binding protein is involved in the $PGF_{2\alpha}$-induced phospholipase D activation.

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