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Effects of Vitamin D_3 on Signaling by Prostaglandin E_2 in Osteoblast-Like Cells

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Abstract We investigated the effects of vitamin D_3 on the signaling pathways by prostaglandin E_2 (PGE_2) in osteoblast-like MC3T3-E1 cells. The pretreatment with 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), an active form of vitamin D_3, significantly inhibited cAMP accumulation induced by 10 μM PGE_2 in a dose-dependent manner in the range between 1 pM and 1 nM. This effect of 1,25-$(OH)_2D_3$ was dependent on the time of pretreatment up to 8 h. 1,25-$(OH)_2D_3$ also inhibited the cAMP accumulation induced by NaF, a GTP-binding protein activator, or forskolin which directly activates adenylate cyclase. On the other hand, 1,25-$(OH)_2D_3$ significantly inhibited PGE_2-induced IP_3 formation in a dose-dependent manner between 10 pM and 1 nM. However, 1,25-$(OH)_2D_3$ had little effect on NaF-induced IP_3 formation. The pretreatment with 24,25-dihydroxyvitamin D_3, an inactive form of vitamin D_3, affected neither cAMP accumulation nor IP_3 formation induced by PGE_2. These results strongly suggest that 1,25-$(OH)_2D_3$ modulates the signaling by PGE_2 in osteoblast-like cells as follows: the inhibitory effect on the cAMP production is exerted at a point downstream from adenylate cyclase and the inhibitory effect on the phosphoinositide hydrolysis is exerted at the point between the PGE_2 receptor and GTP-binding protein, probably G_{i2}.

Key words: vitamin D_3, prostaglandin E_2, cAMP, phosphoinositide, GTP-binding protein, osteoblast

Prostaglandin E_2 (PGE_2) is well known to be a potent bone resorbing agent as an autacoid [Raisz and Martin, 1984; Nijweide et al., 1986]. It has been thought that the actions are mediated through the activation of adenylate cyclase [Atkins and Martin, 1977; Partridge et al., 1981; Hakeda et al., 1985]. It has recently been reported that PGE_2 also stimulates phosphoinositide (PI) hydrolysis in rat osteosarcoma UMR-106 cells [Yamaguchi et al., 1988]. In a previous study [Tokuda et al., 1991], we have shown that a pertussis toxin-sensitive GTP-binding protein, probably G_{i2}, is involved in the signaling between PGE_2 receptor and phospholipase C which hydrolyzes phosphoinositides in cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria [Kodama et al., 1981; Sudo et al., 1983]. So, it is now considered that the effects of PGE_2 are mediated through both cAMP production and PI hydrolysis in osteoblasts.

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1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) is also recognized as a bone resorbing hormone [Raisz et al., 1972; Stern, 1980; DeLuca and Schnoes, 1983]. It is generally considered that the effect of 1,25-$(OH)_2D_3$, as well as other steroid hormones, is exerted through binding to specific intracellular receptors and subsequent activating the expression of gene network [Evans, 1988]. It has been reported that the receptor for 1,25-$(OH)_2D_3$ exists in osteoblasts including osteoblast-like MC3T3-E1 cells [DeLuca and Schnoes, 1983; Kurihara et al., 1986], and that 1,25-$(OH)_2D_3$ increases alkaline phosphatase activity and type I collagen production in MC3T3-E1 cells [Kurihara et al., 1986]. In addition, it has also been shown that 1,25-$(OH)_2D_3$ induces intracellular Ca^{2+} elevation and the formation of inositol trisphosphate (IP_3) within 1 min in ROS 17/2.8 rat osteosarcoma cells [Civitelli et al., 1990; Baran et al., 1991], while 1,25-$(OH)_2D_3$ has been reported to decrease adenylate cyclase activity induced by parathyroid hormone (PTH), also known as a potent bone resorbing hormone in rodent osteoblasts [Chen et al., 1984] and rat osteosarcoma ROS 17/2.8 cells [Catherwood, 1985]. It has also been

reported that 1,25-$(OH)_2D_3$ suppresses PTH-induced prostaglandin E production in osteoblastic cells derived from human bone [MacDonald et al., 1984]. These results suggest that 1,25-$(OH)_2D_3$ modulates signaling pathways by other bone resorbing agents in osteoblasts. However, the precise mechanism of the 1,25-$(OH)_2D_3$ effect on the signaling has not yet been fully elucidated.

In the present study, we examined the effects of 1,25-$(OH)_2D_3$ on the two signaling pathways of PGE_2, cAMP accumulation and PI hydrolysis in osteoblast-like MC3T3-E1 cells. Herein we show that 1,25-$(OH)_2D_3$ modulates the signaling by PGE_2 in osteoblast-like cells as follows: the inhibitory effect on the cAMP accumulation is exerted at a point downstream from adenylate cyclase and the inhibitory effect on PI hydrolysis is exerted at the point between the PGE_2 receptor and GTP-binding protein, probably G_{i2}.

METHODS

Materials

1,25-$(OH)_2D_3$ and 24,25-dihydroxyvitamin D_3 (24,25-$(OH)_2D_3$) dissolved in ethanol were provided by Roche Pharmaceutical Co. (Tokyo, Japan). *myo*-[2-^{3}H] Inositol (81.5 Ci/mmol) was obtained from Amersham Japan (Tokyo, Japan). The cAMP radioimmunoassay kit was kindly provided by Yamasa Shoyu Co. (Chiba, Japan). PGE_2, NaF, and forskolin were obtained from Sigma Chemical Co. (St. Louis, MO). The protein assay reagent kit was purchased from Pierce (Rockford, IL). Other materials and chemicals were obtained from commercial sources. PGE_2 and forskolin were dissolved in ethanol. 3-Isobutyl-1-methylxanthine (IBMX) was dissolved in dimethyl sulfoxide. The maximum concentration of ethanol and dimethyl sulfoxide in the culture medium was 0.1%, and this affected neither the assay for cAMP nor the measurement of IP_3 formation.

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. In experiments for the measurement of the formation of IP_3, the medium was exchanged for 2 ml of inositol-free α-MEM containing 0.3% FCS.

Assay for cAMP

The cultured cells were pretreated with various doses of vitamin D_3 (VD_3) for the indicated periods, and then preincubated with 0.5 mM IBMX for 10 min in 1 ml of an assay buffer (5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4 containing 150 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.8 mM $MgSO_4$, and 1 mM $CaCl_2$) containing 0.01% bovine serum albumin (BSA). After the preincubation, the cells were stimulated by PGE_2, NaF, or forskolin at 37°C. The reaction was terminated by aspirating the medium; then the intracellular cAMP was extracted with 1 ml of 90% *n*-propanol [Herrmann-Erlee et al., 1983]. cAMP in the extracts was measured by a radioimmunoassay kit.

Measurement of the Formation of IP_3

The cultured cells were labeled with *myo*-[2-^{3}H] inositol (3 μCi/dish) for 48 h. After the pretreatment with various doses of VD_3 for the indicated periods, the labeled cells were preincubated with 10 mM LiCl for 10 min in 1 ml of the assay buffer containing 0.01% BSA. The cells were then stimulated by PGE_2 or NaF at 37°C. The reaction was terminated by adding 1 ml of 30% trichloroacetic acid. The acid supernatant was treated with diethyl ether to remove the acid and neutralized with 0.1 M NaOH. The supernatant was applied to a column of Dowex AG1-X8 (100–200 mesh, formate form). To elute inositol monophosphate and inositol bisphosphate, 8 ml of 0.1 M formic acid containing 0.4 M ammonium formate was applied to the column. The radioactive IP_3 was then eluted from the column with 8 ml of 0.1 M formic acid containing 1 M ammonium formate [Berridge et al., 1983, 1984].

Determinations

The radioactivity of ^{3}H samples was determined with a Beckman LS 6000IC liquid scintillation spectrometer. Protein concentrations were determined by using a protein assay reagent kit with BSA as a reference protein.

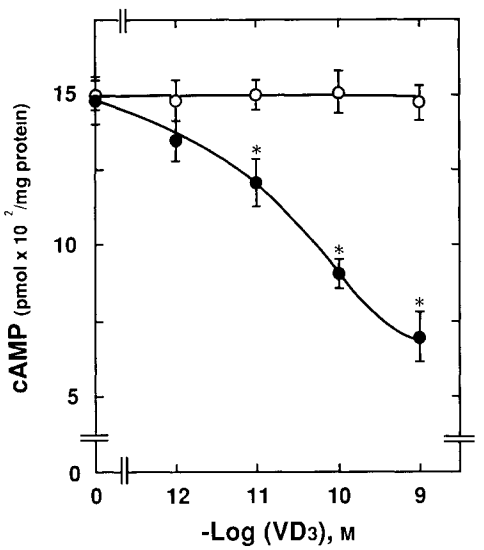

Fig. 1. Effects of VD_3 on PGE_2-induced cAMP accumulation in MC3T3-E1 cells The cultured cells were pretreated with various doses of 1,25-$(OH)_2D_3$ (●) or 24,25-$(OH)_2D_3$ (○) for 8 h, then stimulated by 10 μM PGE_2 for 5 min Values for unstimulated cells have been subtracted from each data point Each value represents the mean ± S D of triplicate determinations Similar results were obtained with two additional and different cell preparations *P < 0 05 compared to the value pretreated with 24,25-$(OH)_2D_3$

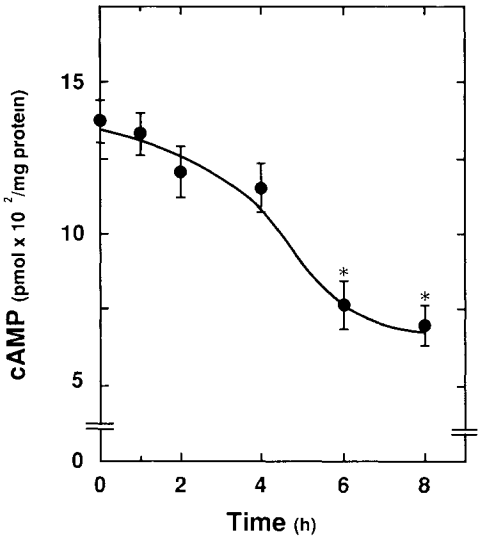

Fig. 2. Effect of 1,25-$(OH)_2D_3$ on PGE_2-induced cAMP accumulation in MC3T3-E1 cells time-course of pretreatment The cultured cells were pretreated with 1 nM 1,25-$(OH)_2D_3$ for the indicated periods, then stimulated by 10 μM PGE_2 for 5 min Values for unstimulated cells have been subtracted from each data point Each value represents the mean ± S D of triplicate determinations Similar results were obtained with two additional and different cell preparations *P < 0 05 compared to the value stimulated simultaneously by PGE_2 and 1,25-$(OH)_2D_3$

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 ± S.D. of triplicate determinations.

RESULTS

Effects of VD_3 on PGE_2-Induced cAMP Accumulation in MC3T3-E1 Cells

In a previous study [Kozawa et al., 1992], we have shown that PGE_2 stimulates cAMP accumulation in a dose-dependent manner in the range between 1 nM and 10 μM in osteoblast-like MC3T3-E1 cells, and that the cAMP accumulation shows the peak at 5 min. Thus, we first examined the effects of VD_3 on cAMP accumulation induced by 10 μM PGE_2 in these cells. The 8-h pretreatment with 1,25-$(OH)_2D_3$, an active form of VD_3 [Stern, 1980; DeLuca and Schnoes, 1983], which by itself had little effect on cAMP accumulation (data not shown), significantly inhibited the PGE_2-induced cAMP accumulation in a dose-dependent manner in the range between 1 pM and 1 nM (Fig. 1). The maximum inhibitory effect of 1 nM 1,25-$(OH)_2D_3$ on the cAMP accumulation was 53%. On the other hand, the pretreatment with 24,25-$(OH)_2D_3$, an inactive form of VD_3 [Stern, 1980; DeLuca and Schnoes, 1983], showed little effect on the PGE_2-induced cAMP accumulation in these cells (Fig. 1). The inhibitory effect of 1,25-$(OH)_2D_3$ was dependent on the time of pretreatment up to 8 h (Fig. 2). The pretreatment with 1,25-$(OH)_2D_3$ had little effect on the protein contents of the cultured cells up to 8 h (0.377 ± 0.015 mg/dish for control and 0.375 ± 0.020 mg/dish for 1 nM 1,25-$(OH)_2D_3$ for 8 h; there was no significant difference from control).

Effects of 1,25-$(OH)_2D_3$ on NaF- or Forskolin-Induced cAMP Accumulation in MC3T3-E1 Cells

To clarify the point exerted by 1,25-$(OH)_2D_3$ in the inhibitory effect on the PGE_2-induced cAMP accumulation, we examined the effects of 1,25-$(OH)_2D_3$ on NaF- or forskolin-induced cAMP accumulation in MC3T3-E1 cells. We have previously reported that NaF, a GTP-binding protein activator [Gilman, 1987], dose dependently induces cAMP accumulation in the range between 5 and 40 mM in these cells [Kozawa et al., 1992]. The cAMP accumulation induced by 40 mM NaF was significantly suppressed by 8 h pretreatment with 1,25-$(OH)_2D_3$ in a dose-dependent manner between 1 pM and 1 nM (Fig.

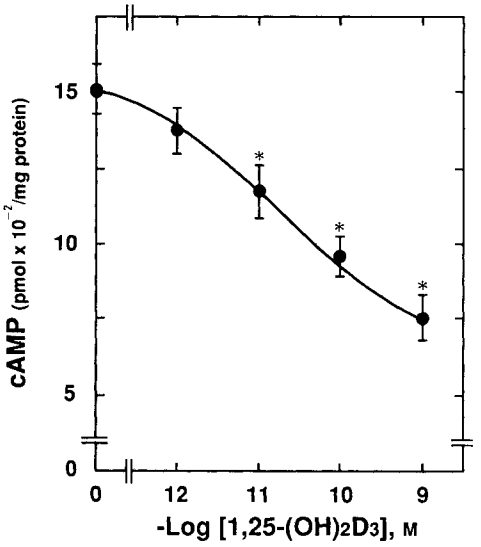

Fig. 3. Effect of 1,25-$(OH)_2D_3$ on NaF-induced cAMP accumulation in MC3T3-E1 cells. The cultured cells were pretreated with various doses of 1,25-$(OH)_2D_3$ for 8 h, then stimulated by 40 mM NaF for 5 min. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *P < 0.05 compared to control.

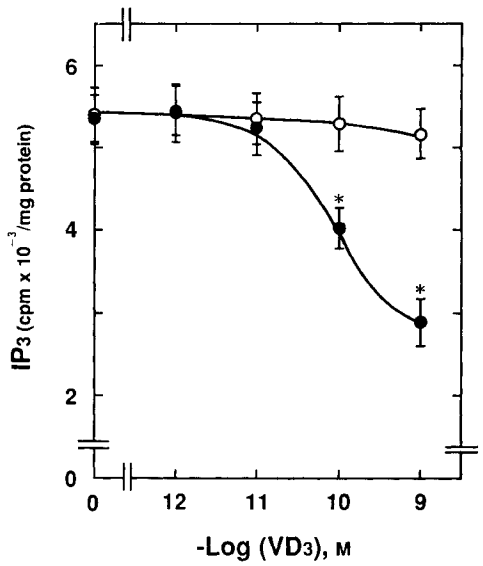

Fig. 4. Effects of VD_3 on PGE_2-induced IP_3 formation in MC3T3-E1 cells. The [^{3}H]inositol-labeled cells were pretreated with various doses of 1,25-$(OH)_2D_3$ (●) or 24,25-$(OH)_2D_3$ (○) for 8 h, then stimulated by 10 μM PGE_2 for 5 min. Values for unstimulated cells have been subtracted from each data point. Each value represents the mean ± S.D. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. *P < 0.05 compared to the value pretreated with 24,25-$(OH)_2D_3$.

3). The cAMP accumulation induced by 50 μM forskolin, which directly activates adenylate cyclase [Seamon and Daly, 1981], was also inhibited by 1,25-$(OH)_2D_3$ in these cells (3,424 ± 72 pmol/mg protein for control and 2,326 ± 76 pmol/mg protein for 1 nM 1,25-$(OH)_2D_3$–treated cells; the cells were stimulated by 50 μM forskolin for 5 min).

Effects of VD_3 on PGE_2-Induced IP_3 Formation in MC3T3-E1 Cells

Previously, we have shown that PGE_2 stimulates PI hydrolysis in a dose-dependent manner in the range between 1 nM and 10 μM in MC3T3-E1 cells, and that PGE_2-induced IP_3 formation reaches a plateau almost within 10 min and sustains it up to 30 min [Kozawa et al., 1992]. So, we next examined the effects of VD_3 on the IP_3 formation induced by 10 μM PGE_2 in these cells. The 8 h pretreatment with 1,25-$(OH)_2D_3$, which by itself had little effect on IP_3 formation (data not shown), significantly inhibited the PGE_2-induced IP_3 formation in a dose-dependent manner in the range between 10 pM and 1 nM (Fig. 4). The maximum inhibitory effect of 1 nM 1,25-$(OH)_2D_3$ on the IP_3 formation was 46%. On the other hand, 24,25-$(OH)_2D_3$ showed little effect on the IP_3 formation induced by PGE_2 in these cells (Fig. 4). The inhibitory effect of 1,25-$(OH)_2D_3$ was dependent on the time of pretreatment up to 8 h (Fig. 5).

Effect of 1,25-$(OH)_2D_3$ on NaF-Induced IP_3 Formation in MC3T3-E1 Cells

In a previous study [Tokuda et al., 1991], we have shown that NaF mimicks PGE_2 by activating the formation of inositol phosphates in MC3T3-E1 cells, and that the effect of 20 mM NaF is very similar to that of 10 μM PGE_2. We have also demonstrated that a pertussis toxin-sensitive GTP-binding protein, probably G_{i2}, is involved in the signaling between PGE_2 receptor and phospholipase C [Tokuda et al., 1991]. To clarify the point exerted by 1,25-$(OH)_2D_3$ in the inhibitory effect on the PGE_2-induced PI hydrolysis, we examined the effect of 1,25-$(OH)_2D_3$ on the NaF-induced IP_3 formation. NaF (20 mM)-induced IP_3 formation was little affected by 8-h pretreatment with 1,25-$(OH)_2D_3$ in these cells (Fig. 6).

DISCUSSION

In the present study, we first examined the effects of VD_3 on the PGE_2-induced cAMP accumulation in osteoblast-like MC3T3-E1 cells and showed that not 24,25-$(OH)_2D_3$, an inactive form

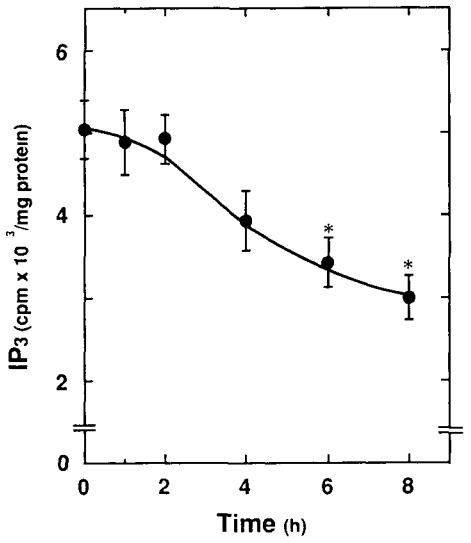

Fig. 5. Effect of 1,25-$(OH)_2D_3$ on PGE_2-induced IP_3 formation in MC3T3-E1 cells time-course of pretreatment The [^{3}H]inositol-labeled cells were pretreated with 1 nM 1,25-$(OH)_2D_3$ for the indicated periods, then stimulated by 10 μM PGE_2 for 5 min Values for unstimulated cells have been subtracted from each data point Each value represents the mean ± S D of triplicate determinations Similar results were obtained with two additional and different cell preparations *P < 0 05 compared to the value stimulated simultaneously by PGE_2 and 1,25-$(OH)_2D_3$

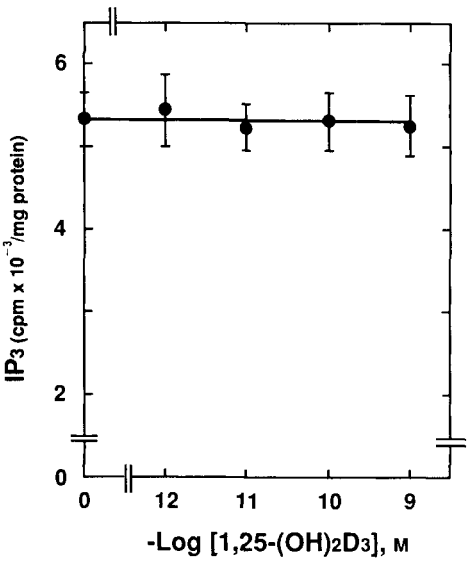

Fig. 6. Effect of 1,25-$(OH)_2D_3$ on NaF-induced IP_3 formation in MC3T3-E1 cells The [^{3}H]inositol-labeled cells were pretreated with various doses of 1,25-$(OH)_2D_3$ for 8 h, then stimulated by 20 mM NaF for 10 min Values for unstimulated cells have been subtracted from each data point Each value represents the mean ± S D of triplicate determinations Similar results were obtained with two additional and different cell preparations

of VD_3, but 1,25-$(OH)_2D_3$, an active form of VD_3 [Stern, 1980; DeLuca and Schnoes, 1983] markedly inhibited the cAMP accumulation in a dose-dependent manner in these cells. It is well established that G_s is involved in the signaling between the receptor and adenylate cyclase [Gilman, 1987]. To clarify the focus of 1,25-$(OH)_2D_3$ in the inhibitory effect on the PGE_2-induced cAMP accumulation, therefore, we examined the effects of 1,25-$(OH)_2D_3$ on NaF- or forskolin-induced cAMP accumulation in MC3T3-E1 cells. We demonstrated that 1,25-$(OH)_2D_3$ significantly reduced the cAMP accumulation induced by these two agents. Since NaF is an activator of GTP-binding protein [Gilman, 1987] and forskolin is known to activate adenylate cyclase directly [Seamon and Daly, 1981], these results suggest that the inhibitory effect of 1,25-$(OH)_2D_3$ on the PGE_2-induced cAMP production is exerted at a point downstream of the signaling from adenylate cyclase. It has been reported that PTH-induced cAMP accumulation is reduced by 1,25-$(OH)_2D_3$ in cultured rodent bone cells [Chen et al., 1984] and rat osteosarcoma ROS 17/2.8 cells [Catherwood, 1985]. In ROS 17/2.8 cells, it has been shown that 1,25-$(OH)_2D_3$ had little effect on the cAMP accumulation induced by cholera toxin, an activator for G_s, or forskolin [Catherwood, 1985], and that the inhibitory effect of 1,25-$(OH)_2D_3$ results from the reduction of PTH receptor number [Titus et al., 1991]. These discordances may be due to the difference of cell species or some experimental conditions.

We next examined the effects of VD_3 on the PGE_2-induced PI hydrolysis in MC3T3-E1 cells and showed that not 24,25-$(OH)_2D_3$ but 1,25-$(OH)_2D_3$ also reduced the IP_3 formation induced by PGE_2. To our knowledge, this is probably the first report on an inhibitory effect of 1,25-$(OH)_2D_3$ on PI hydrolysis in osteoblasts. In a previous study [Tokuda et al., 1991], we have demonstrated that PGE_2-induced PI hydrolysis is mediated through a pertussis toxin-sensitive GTP-binding protein, probably G_{i2}, in MC3T3-E1 cells. Therefore, to clarify the focus of 1,25-$(OH)_2D_3$ in the inhibitory effect, we examined the effect of 1,25-$(OH)_2D_3$ on NaF-induced IP_3 formation and showed that 1,25-$(OH)_2D_3$ had little effect on the IP_3 formation induced by NaF in these cells. From this result, it seems unlikely that 1,25-$(OH)_2D_3$ affects post-G_{i2} signaling in the PGE_2-induced PI hydrolysis. It is well known that PGE_2 receptors are pharmacologically subdivided into three subtypes, EP_1, EP_2, and EP_3

[Coleman et al., 1989], and these subtypes are considered to be different in their signal transduction; they are presumed to be coupled to stimulation of phospholipase C, and stimulation and inhibition of adenylate cyclase, respectively [Yokohama et al., 1988; Chen et al., 1988; Sonnenburg and Smith, 1988; Coleman et al., 1989]. In MC3T3-E1 cells, existence of two classes of PGE_2 receptors mediating the stimulation of adenylate cyclase and phospholipase C has recently been reported [Toriyama et al., 1992]. Thus, it is most likely that the inhibitory effect of 1,25-$(OH)_2D_3$ on PGE_2-induced PI hydrolysis is exerted at the point between the PGE_2 receptor and GTP-binding protein, probably G_{i2}.

We showed here that 24,25-$(OH)_2D_3$ had little effect on either cAMP accumulation or IP_3 formation induced by PGE_2 in MC3T3-E1 cells. Since it is well accepted that 24,25-$(OH)_2D_3$ is an inactive metabolite of VD_3 in bone resorbing activity [Stern, 1980; DeLuca and Schnoes, 1983], it is possible that the effects of 1,25-$(OH)_2D_3$ shown here play a crucial role in bone metabolism. In addition, we have recently reported that 17 β-estradiol markedly inhibits PGE_2-induced PI hydrolysis without affecting PGE_2-induced cAMP production in these cells [Tokuda et al., 1992]. This significant difference between the effects of 1,25-$(OH)_2D_3$ and that of 17 β-estradiol on PGE_2 signaling may reflect the different actions of these two steroid hormones on bone metabolism.

In conclusion, our results strongly suggest that 1,25-$(OH)_2D_3$ modulates the signaling by PGE_2 in osteoblast-like cells as follows: the inhibitory effect on the cAMP production is exerted at a point downstream from adenylate cyclase and the inhibitory effect on PI hydrolysis is exerted at the point between the PGE_2 receptor and GTP-binding protein, probably G_{i2}.

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