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Prostaglandin E_2 Stimulates the Formation of Mineralized Bone Nodules by a cAMP-Independent Mechanism in the Culture of Adult Rat Calvarial Osteoblasts

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Abstract The effects of prostaglandin E_2 (PGE_2) on the proliferation and differentiation of osteoblastic cells were studied in osteoblast-like cells isolated from adult rat calvaria. Treatment of the cells with PGE_2 within the concentration range 10^{-8}–10^{-5} M resulted in a dose-dependent increase in alkaline phosphatase (ALP) activity, [^{3}H]proline incorporation into collagenase-digestible protein, and mineralized bone nodule (BN) formation, as well as a dose-dependent decrease in [^{3}H]thymidine incorporation into the cells. PGE_2 also caused a dose-dependent increase in the intracellular cyclic adenosine monophosphate (cAMP) content, with a maximal effective concentration of 10^{-5} M; this effect of PGE_2 was mimicked by forskolin, an adenylate cyclase activator. The treatment of adult calvarial cells with forskolin decreased BN formation, ALP activity, and collagen synthesis. These results suggested that cAMP does not have a stimulatory, but rather a suppressive, effect on the differentiation of adult rat calvarial cells. A time-course study of cAMP accumulation showed that both PGE_2- and forskolin-induced cAMP reached a maximum at 5 min after the treatment, but the former rapidly returned to the basal level by 40 min, while the latter declined slowly and was still at 70% of the maximal level at 60 min, suggesting that PGE_2 activates phosphodiesterase as well as adenylate cyclase. The presence of N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), a calmodulin antagonist, reduced the rate of degradation of cAMP formed after PGE_2 treatment, suggesting the involvement of calmodulin in the activation of phosphodiesterase. However, PGE_2 also caused the production of inositol 1,4,5-triphosphate (IP_3) and an elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), both of which peaked at 15 s and returned to the basal level within 1 min. Submaximal responses of the IP_3 production and the $[Ca^{2+}]_i$ elevation to PGE_2 were obtained at 10^{-5} M. W-7 decreased both basal and PGE_2-induced ALP activity, collagen synthesis and BN formation, indicating the involvement of Ca^{2+}/calmodulin-dependent protein kinase in the PGE_2-induced differentiation of calvarial cells. From these results, we concluded that PGE_2 inhibits the proliferation and stimulates the differentiation of calvarial osteoblasts by elevating the $[Ca^{2+}]_i$ through the activation of a phosphoinositide turnover, but not via an activation of adenylate cyclase. We also found that BN formation varies, depending on the time of PGE_2 addition, suggesting that responsiveness of the cells to PGE_2 may change during the culture period. J. Cell. Biochem. 73:36–48, 1999.

Key words: osteoblasts; cAMP; phosphoinositide turnover; calmodulin; prostaglandin E_2 (PGE_2); mineralized bone nodule

Prostaglandins (PGs) are considered important local factors that modulate the metabolism of bone tissue [Nijweide et al., 1986; Norrdin et al., 1990]. Among PGs, prostaglandin E_2 (PGE_2) is a major eicosanoid produced by osteoblastic cells [Partridge et al., 1985; Yokota et al., 1986]. PGE_2 was first shown to be a potent bone-resorbing agent [Klein and Raisz, 1970; Dietrich et al., 1975]; subsequent studies demonstrated that it is also a modulator of osteoblast function [Chyun and Raisz, 1984; Jee et al., 1985; Hakeda et al., 1985]. PGE_2-induced responses in osteoblastic cells have been most extensively studied in a clonal osteoblastic cell line, MC3T3-E1 cells, in which this eicosanoid inhibits DNA synthesis and stimulates differentiation markers such as alkaline phosphatase (ALP) activity and collagen synthesis at low

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concentrations (approximately 10^{-8}–10^{-7} M), while it stimulates DNA synthesis and inhibits differentiation markers at high concentrations (approximately 10^{-6}–10^{-5} M) [Hakeda et al., 1986, 1987a]. The dual effects of PGE_2 have been ascribed to the difference in the signal transduction system leading to the cellular responses at high and low concentrations. The differentiative effect of PGE_2 at low concentrations is mediated predominantly by the elevation of intracellular cAMP as a consequence of the activation of adenylate cyclase, while the proliferative effect is mediated predominantly by the stimulation of phosphoinositide (PI) turnover by the activation of PI-specific phospholipase C (PI-PLC) [Hakeda et al., 1987b]. The action of PGE_2 also seems to vary depending on the cell type. PGE_2 stimulates the cell replication and protein synthesis in cell cultures obtained from chick tibia [Nagai, 1989], but not in osteoblast-enriched cells from fetal rat calvaria [Centrella et al., 1994]. Experiments with organ cultures of fetal rat calvaria showed that PGE_2 causes stimulation of DNA synthesis in the periosteum and a suppression of collagen synthesis in the central bone, probably reflecting the difference in cell population in each area of bone [Raisz and Koolemans-Beynen, 1974]. The in vivo administration of PGE_2 to rats has been reported to cause age-dependent effects on bone metabolism [Jee et al., 1985, 1990]. Within this context, it is of interest to determine how PGE_2 behaves in osteoblastic cells from adult animals. A culture model of mineralized bone nodule (BN) formation by adult rat calvarial cells was recently developed by Kato et al. [1995]. The present study was undertaken to determine whether PGE_2 induces functional alterations in adult calvarial cells similar to those observed in MC3T3-E1 cells. We found that PGE_2 stimulates the differentiation and inhibits the proliferation of adult rat calvarial cells within a concentration range of 10^{-8}–10^{-5} M and that cAMP does not mediate the stimulative effect of PGE_2 on cell differentiation, a finding that is different from that reported for MC3T3-E1 cells. We also obtained the results suggesting that the time of PGE_2 addition may be an important factor that affects cellular responses.

MATERIALS AND METHODS

Materials

PGE_2, forskolin, W-7, 3-(isobutyl)-1-methylxantine (IBMX), calphostin C, and 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemical Co. (St. Louis, MO). KT5720 and dibutyryl-cAMP were from Biomol (Plymouth Meeting, PA). Fetal bovine serum (FBS), phenol red-free F-12 medium and α-minimum essential medium (α-MEM) and trypsin were products of Gibco-BRL (Rockville, MD). Kanamycin, ascorbic acid, collagenase and β-glycerophosphate were obtained from Wako Pure Chemical Industries (Tokyo, Japan). [2,3-^{3}H]proline and [methyl-^{3}H]thymidine were from DuPont (Wilmington, DE).

Cell Culture

Cells enriched for osteoblast phenotype were enzymatically isolated from the calvaria of 25- to 35-week-old female Wistar rats (CLEA, Tokyo, Japan) according to the method of Bellows et al. [1986], modified by Kato et al. [1995]. Briefly, after five sequential digestions of calvaria with a mixture of collagenase and trypsin, the released cells from the last three digestion intervals were grown in F-12 medium containing 10% fetal bovine serum (FBS). After reaching confluence, the cells were collected by a trypsin treatment, seeded in 4-well dishes at 2×10^3 cells/cm^2 in the same medium, and cultured for 4 days (designated as the proliferation period, P1–P4). At the end of day P4, the medium was changed to α-MEM supplemented with 10% FBS, 2 mM β-glycerophosphate, and ascorbic acid (0.1 mg/ml), and the cells were maintained for a further 18 days (designated as the mineralization period, M1–M18).

Determination of Markers for Cell Proliferation and Differentiation

For the determination of ALP activity, collagen synthesis, BN formation, and DNA synthesis, cells were incubated in serum-free F-12 medium for 24 h on day P4, with appropriate doses of PGE_2 present in the medium during the last 6 h; incubation was continued in PGE_2-free α-MEM medium as described above. An ALP assay was performed at the beginning of day M2 according to the method of Lowry et al. [1954], using p-nitrophenylphosphate as a substrate. For the assay of collagen synthesis, cells

were incubated in serum-free α-MEM medium containing [2,3-^{3}H]proline (1.25 μCi/ml) for 5 h at the beginning of day M2. The incorporation of radioactivity into collagenase-digestible protein was measured as described by Peterkofsky and Diegelman [1971]. For quantification of BN, BN were visualized by von Kossa's stain [Bharagava et al., 1988], and the total nodule area and the number of nodules were assessed on day M18 using a colony counter (BMS-400, Toyo Sokki, Tokyo). For the determination of DNA synthesis, the cells were incubated for 3 h in serum-free α-MEM medium containing [^{3}H]thymidine (1.25 μCi/ml) at the beginning of day M1, and the incorporation of radioactivity into DNA was measured.

Determination of cAMP, Ca^{2+}, and Inositol Triphosphate Formation

For the determination of intracellular cAMP, cells were incubated in serum-free F12 medium for 18 h at the beginning of day P4 and treated with 10^{-5} M IBMX for 20 min. The cells were stimulated with 10^{-6} M PGE_2 for 5 min, and the reaction was terminated by removal of the medium, followed by the addition of 0.5 ml of cold 90% 1-propanol. The extracts were dried and resolubilized in 0.05 M sodium acetate, and cAMP contents were determined with the use of a radioimmunoassay (RIA) kit (Yamasa, Chiba, Japan). For the determination of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), cells were incubated in serum-free F12 medium for 18 h and then treated with 10^{-6} M PGE_2 for 15 s on day P4. The $[Ca^{2+}]_i$ was measured according to the method of Grynkiewicz et al. [1985]. Briefly, the cells were incubated with 10 μM fura-2/AM for 2 h at 37°C in serum-free F-12 medium to hydrolyze the acetoxymethyl ester of fura-2 completely, and then washed twice with the same medium. The ratio of fluorescence intensity excited at 340 nm and 380 nm (500-nm emission) was measured with a Cyto Fluor II automatic plate-reading fluorometer (PerSeptive Biosystems, Tokyo, Japan). For the determination of the inositol 1,4,5-triphosphate (IP_3) generation, cells were incubated in inositol- and serum-free medium containing 0.01% bovine serum albumin (BSA) for 18 h on day P4. During the last 1 h of the incubation, 10 mM LiCl was included in the medium. The cells were treated with 10^{-6} M PGE_2 for 0.5 min. The reaction was terminated by the removal of the medium, and the cells were scraped into 1 ml of 10% trichloroacetic acid (TCA). The dishes were washed twice with 1 ml of 10% TCA, and the combined solution was mixed with 1 ml of water-saturated diethyl ether. The water layer was applied to a column of anion-exchange resin (AG 1-X8). IP_3 was eluted from the column with 0.1 M formic acid containing 1 M ammonium formate, and the amount of IP_3 was determined with the use of an RIA kit (Amersham, Tokyo, Japan).

Statistical Methods

Data were analyzed by Student's t-test or by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. $P < 0.01$ was considered significant. All data are presented as the mean ±SD of triplicate determinations.

RESULTS

To determine the effect of PGE_2 on the proliferation and differentiation, we treated adult rat calvarial cells with various concentrations of PGE_2 for 6 h on day P4 after an 18-h serum starvation period (Fig. 1). PGE_2 stimulated both the area (Fig. 1A; $EC_{50} = 7.9 \times 10^{-8}$ M) and the number (Fig. 1B; $EC_{50} = 5.0 \times 10^{-8}$ M) of BN in a dose-dependent manner within a concentration range of 10^{-8}–10^{-5} M, with maximal increases of 2.5-fold and 2.0-fold, respectively, over those of the controls. The typical profiles of BN visualized by von Kossa's stain are shown in Figure 2. PGE_2 increased the ALP activity (Fig. 1C; $EC_{50} = 5.3 \times 10^{-8}$ M) and [^{3}H]proline incorporation into collagenase-digestible protein (Fig. 1D; $EC_{50} = 5.0 \times 10^{-8}$ M) with a dose responsiveness similar to the stimulation of BN formation, and the maximal values, obtained at the PGE_2 concentration of 10^{-5} M, were 2.1- and 2.7-fold, respectively, higher than the control values. These results indicate that PGE_2 has a stimulative effect on the differentiation of adult rat calvarial cells. By contrast, PGE_2 within the same concentration range decreased the [^{3}H]thymidine incorporation into the cells to approximately 50% that in the control cultures (Fig. 1E; $IC_{50} = 2.0 \times 10^{-7}$ M). Although PGE_2 has been reported to inhibit the differentiation and stimulate the proliferation of MC3T3-E1 cells within the high concentration range [Hakeda et al., 1986, 1987a], no such effects were observed under our experimental conditions.

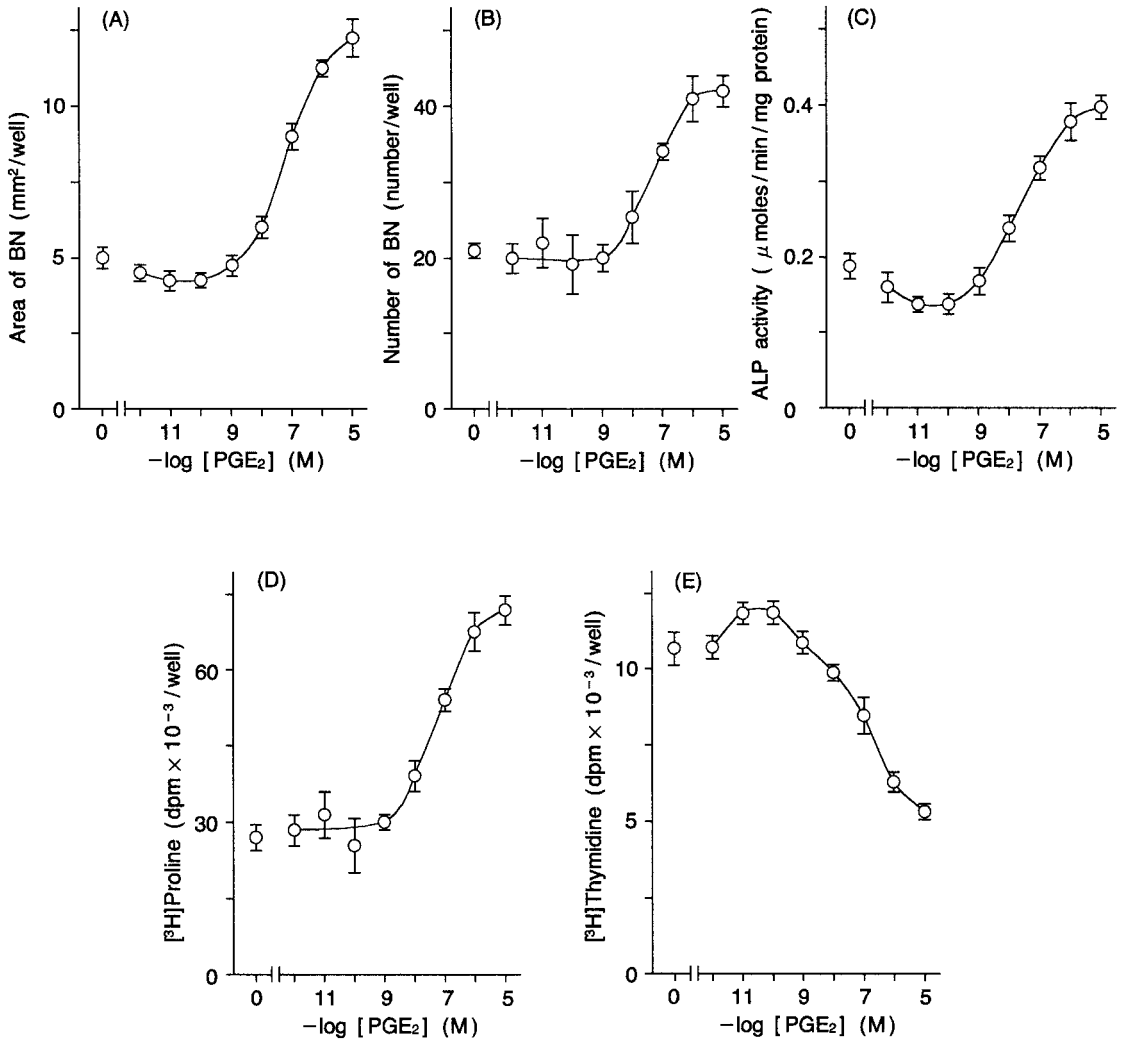

Fig. 1. Effect of various concentrations of PGE_2 on BN formation, ALP activity, collagen synthesis, and DNA synthesis in the culture of adult rat calvarial cells. The cells were treated with various concentrations of PGE_2 during the last 6 h of day P4. ALP activity **(C)**, [^{3}H]proline incorporation into collagenase-digestible protein **(D)**, and [^{3}H]thymidine incorporation into the cells **(E)** were measured at the beginning of day M2. The area **(A)** and number **(B)** of BN were measured on day M18. Other conditions were the same as those described under Materials and Methods. Each point represents the mean ±SD of four cultures. The experiment was repeated twice, and the results were essentially the same as those depicted.

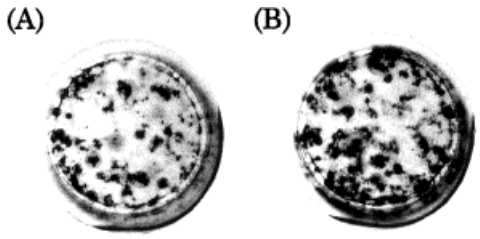

Fig. 2. Typical profiles of BN visualized with von Kossa's stain. **A:** control. **B:** 10^{-6}M PGE_2.

As it is believed that PGE_2 exerts differentiative effects on osteoblast-like cells through the activation of adenylate cyclase [Hakeda et al., 1986, 1987a], we examined the effect of PGE_2 on the production of cAMP by calvarial cells pretreated with IBMX, a phosphodiesterase inhibitor (Fig. 3A). PGE_2 increased cAMP concentration in a dose-related fashion ($EC_{50} = 3.8 \times 10^{-8}$ M), with a maximal increase obtained at 10^{-5} M 45-fold higher than the control value. Forskolin, an adenylate cyclase activator, gave a similar profile of dose-dependent cAMP production in the presence of IBMX within the

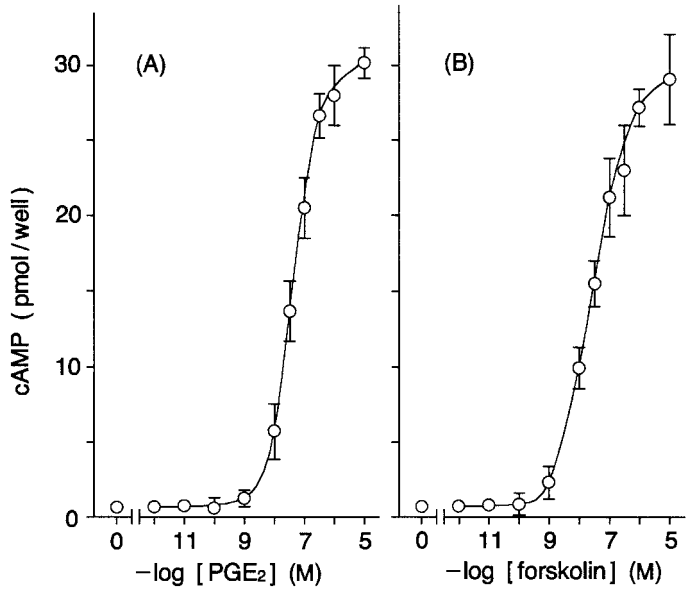

Fig. 3. Effect of various concentrations of PGE_2 and forskolin on cAMP formation in adult rat calvarial cells. After pretreatment with 10^{-5} M IBMX for 20 min, the cells were stimulated with the indicated concentrations of PGE_2 **(A)** or forskolin **(B)** on day P4. Intracellular cAMP was determined by a radioimmunoassay as described under Materials and Methods. Each value represents the mean ±SD of three cultures. The experiment was repeated twice, and the results were essentially the same as those depicted.

range of 10^{-9}–10^{-5} M ($EC_{50} = 2.7 \times 10^{-8}$ M), with the maximal value obtained at 10^{-5} M 40-fold that of the control (Fig. 3B). Contrary to our expectations, forskolin, within this concentration range, inhibited both the area (Fig. 4A; $IC_{50} = 3.3 \times 10^{-8}$ M) and the number (Fig. 4B) of BN, ALP activity (Fig. 4C; $IC_{50} = 6.3 \times 10^{-8}$ M), and [^{3}H]proline incorporation into collagenase-digestible protein (Fig. 4D; $IC_{50} = 7.4 \times 10^{-8}$ M) in a dose-dependent manner; the presence of IBMX potentiated these suppressive effects of forskolin, with approximate IC_{50} values of 8.4×10^{-9}, 3.3×10^{-8} and 1.8×10^{-8} M for the area of BN, ALP activity, and [^{3}H]proline incorporation into collagenase-digestible protein, respectively. By contrast, forskolin increased [^{3}H]thymidine incorporation into the cells ($EC_{50} = 3.5 \times 10^{-8}$ M), and IBMX potentiated this effect ($EC_{50} = 3.2 \times 10^{-8}$ M) (Fig. 4E). Treatment of the cells with 10^{-4} M dibutyryl-cAMP (db-cAMP), a cell-permeable cAMP analogue, decreased the area (control: 4.83 ± 0.26 mm^2/well, db-cAMP: 2.76 ± 0.45 mm^2/well) and the number of BN (control: 23.4 ± 2.2, db-cAMP: 18.3 ± 3.1) and ALP activity (control: 0.193 ± 0.009 µmol/min/mg, db-cAMP: 0.105 ± 0.011 µmol/min/mg), and increased [^{3}H]thymidine incorporation into the cells (control: 9,630 ± 320 dpm/well, db-cAMP: 15,200 ± 500 dpm/well). In addition, treatment of 10^{-6} M KT5720, a selective inhibitor of protein kinase A (PKA), increased the basal level and PGE_2-induced level of the area of BN (control: 4.95 ± 0.21 mm^2/well, PGE_2: 12.1 ± 0.72 mm^2/well, KT5720: 6.05 ± 0.42 mm^2/well, KT5720+PGE_2: 16.8 ± 1.21 mm^2/well). These results seem to indicate that cAMP acts as a suppressor of differentiation and a stimulator of cell proliferation.

Since PGE_2 stimulated cell differentiation in spite of its ability to induce the production of cAMP, which was inhibitory to cell differentiation as described above, we next examined the time course of the level of cAMP after PGE_2 treatment in the absence of IBMX to determine whether the cyclic nucleotide also accumulates under the physiological condition. PGE_2 at 10^{-6} M caused a rapid increase in cAMP and its level peaked at 5 min, followed by a sharp decrease to near the basal value by 40 min (Fig. 5). Only a slight increase could be detected at the PGE_2 concentration of 10^{-8} M, and no change was observed at 10^{-10} M (data not shown). Forskolin also elevated the cAMP level, with maximal accumulation at 5 min, but the subsequent decrease was more gradual than that observed with PGE_2 (10^{-6} M); the level of cAMP was still 40% of the maximal value 60 min after the stimulation (Fig. 5). These results could be ex-

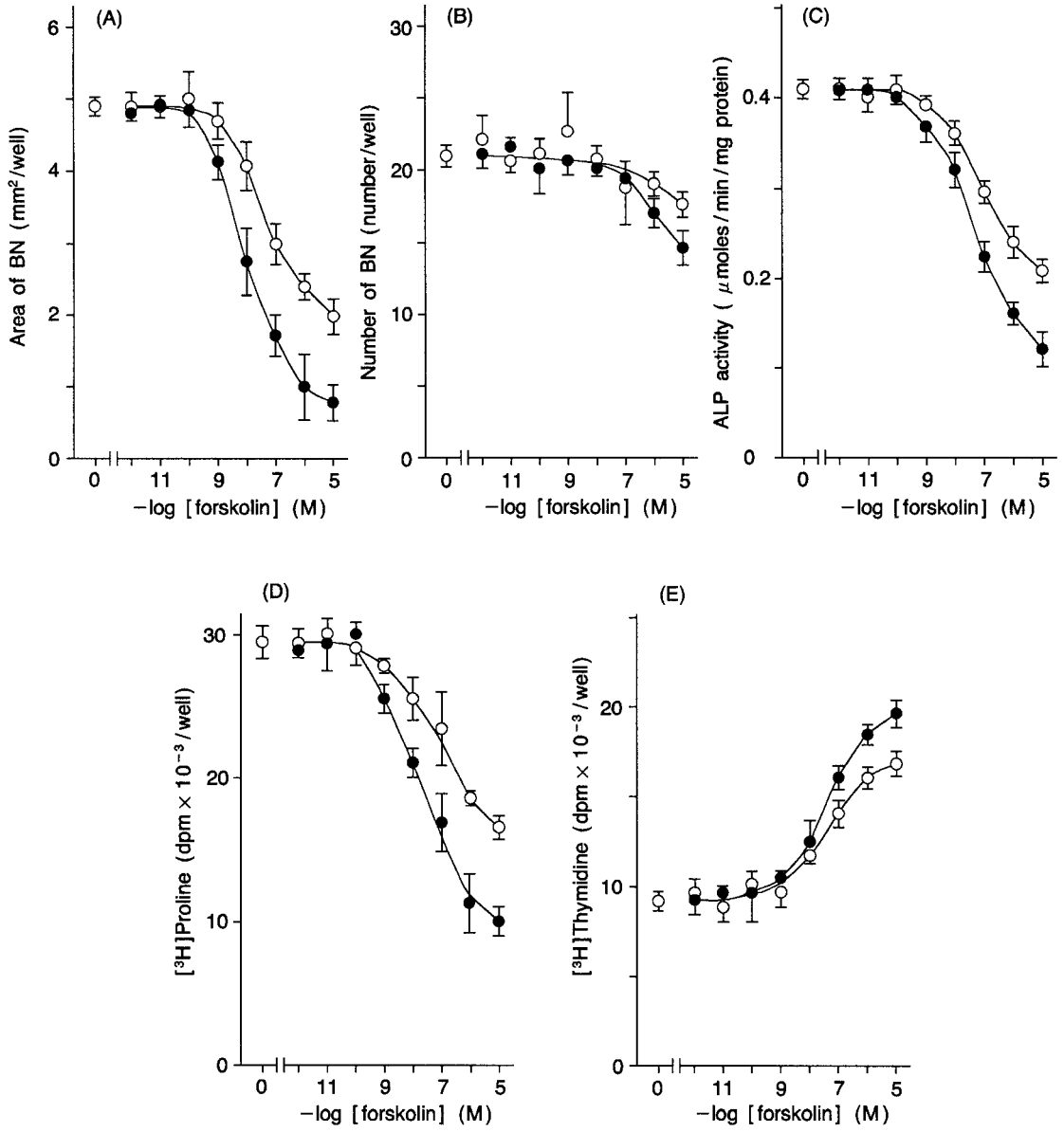

Fig. 4. Effect of forskolin on BN formation, ALP activity, collagen synthesis, and DNA synthesis in adult rat calvarial cells. After preincubation with (●) or without (○) IBMX (10^{-6} M) for 15 min, the cells were treated with various concentrations of forskolin for 6 h on day P4. ALP activity **(C)**, [^{3}H]proline incorporation into collagenase-digestible protein **(D)** and [^{3}H]thymidine incorporation into the cells **(E)** were measured at the beginning of day M2. The area **(A)** and number **(B)** of BN were measured on day M18. Other conditions were the same as those described under Materials and Methods. Each value represents the mean ±SD of three cultures. The experiment was repeated twice, and the results were essentially the same as those depicted.

plained by assuming that PGE_2 activates adenylate cyclase to increase cAMP production and that it also activates phosphodiesterase to degrade newly formed cAMP. To test this assumption, we used W-7 (a calmodulin inhibitor) to block the activation of phosphodiesterase, as calmodulin is reported to be involved in the agonist-induced activation of phosphodiesterase. When PGE_2 was added in the presence of W-7, the maximal level of cAMP obtained 5 min after the treatment was 23% higher than that obtained in the absence of W-7 and the rate of decline in cAMP level was much slower, supporting our assumption (Fig. 5). In addition, the rate of cAMP degradation was also slow when adenylate cyclase was activated by forskolin, which does not affect the activity of phosphodiesterase. The time course of the level of cAMP

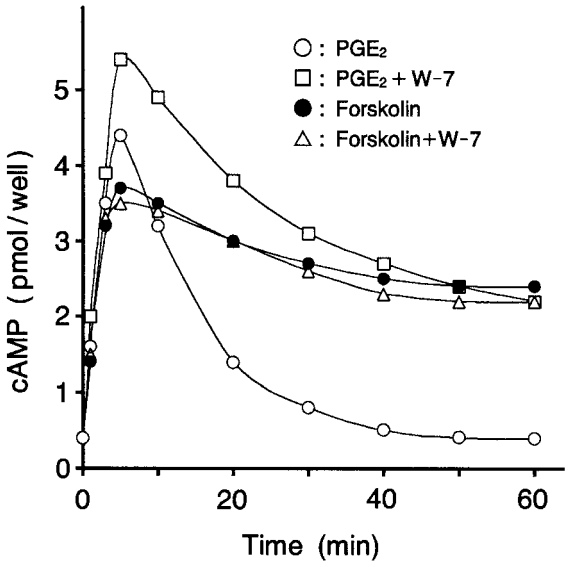

Fig. 5. Time course of the effect of PGE_2 and forskolin on the accumulation of cAMP in adult rat calvarial cells. The cells were treated with 10^{-6} M PGE_2 ○, □) or 10^{-6} M forskolin (●) with (□) or without (○, ●) 10^{-6} M W-7 in the absence of IBMX on day P4. The method used for the measurement of intracellular cAMP and other conditions were the same as those described under Materials and Methods. Each value represents the mean ±SD of three cultures. The experiment was repeated twice, and the results were essentially the same as those depicted.

after the forskolin treatment was not affected by the addition of W-7 (Fig. 5). From these results, it is conceivable that, although PGE_2 induces cAMP production, it does not act as an inhibitor of cell differentiation because of its rapid degradation.

Because PGE_2-induced stimulation of calvarial cell differentiation cannot be explained by the increase in the cAMP level, there should be another signal transduction pathway leading to cell differentiation. A possible candidate for that may be a PI-PLC/Ca^{2+} system. We therefore examined the effect of PGE_2 on the PI turnover in adult rat calvarial cells. Upon the addition of PGE_2 to cells, the level of IP_3 sharply increased, reaching a maximum at 15 s and returning to near the basal level at 1 min, indicating the stimulation of phosphatidylinositol diphosphate (PIP_2) hydrolysis by PGE_2 (Fig. 6A). By varying the concentration of PGE_2, we found that the maximal stimulation of IP_3 formation was obtained at 10^{-6} M–10^{-5} M (Fig. 6B, $EC_{50} = 8.4 \times 10^{-8}$ M). The time course (Fig. 6C) and dose dependency (Fig. 6D) of the $[Ca^{2+}]_i$ closely matched those of IP_3 formation; i.e., it peaked at around 15 s, and the maximal increase was obtained at a PGE_2 concentration of 10^{-6}–10^{-5} M ($EC_{50} = 2.8 \times 10^{-7}$ M), suggesting that the increase in $[Ca^{2+}]_i$ was due to a Ca^{2+} release from intracellular stores.

To test the possibility that the PGE_2-induced cell responses are mediated through calmodulin activated as a consequence of the increase in $[Ca^{2+}]_i$, the effects of W-7 on the differentiation and proliferation of calvarial cells were examined (Fig. 7). W-7 strongly decreased both the basal level and the PGE_2-induced level of BN formation, in both the area (Fig. 7A; IC_{50} for basal level = 3.3×10^{-8} M, IC_{50} for PGE_2-induced level = 2.1×10^{-8} M) and the number (Fig. 7B; IC_{50} for basal level = 1.0×10^{-8} M, IC_{50} for the PGE_2-induced level = 8.4×10^{-9} M), the ALP activity (Fig. 7C; IC_{50} for basal level = 3.2×10^{-8} M, IC_{50} for PGE_2-induced level = 1.2×10^{-8} M), and the incorporation of [^{3}H]proline into collagenase-digestible protein (Fig. 7D; IC_{50} for basal level = 8.4×10^{-9} M, IC_{50} for PGE_2-induced level = 3.5×10^{-8} M). By contrast, this calmodulin inhibitor resulted in a dose-dependent increase in [^{3}H]thymidine incorporation by the cells (Fig. 7E). These results indicate that both the stimulation of differentiation and the suppression of proliferation by PGE_2 are mediated by Ca^{2+}/calmodulin in adult rat calvarial cells.

Diacylglycerol (DAG), in addition to IP_3, is the product of the PI-PLC-catalyzed hydrolysis of PIP_2 and is also known to act as a second messenger of signal transduction by activating protein kinase C (PKC). We therefore examined whether PKC mediates the differentiative effect of PGE_2 in adult rat calvarial cells using TPA, an activator of PKC, and calphostin C, an inhibitor of PKC. No significant change was produced in the area of BN by the treatment of the cells with 10^{-8} M TPA or 10^{-8} M calphostin C (control: 4.78 ± 0.33 mm^2/well, TPA: 4.28 ± 0.50 mm^2/well, calphostin C: 4.93 ± 0.39 mm^2/well). These results indicate that PKC is not involved in the PGE_2-induced differentiation of adult rat calvarial cells.

The effects of PGE_2 on the proliferation and differentiation of adult calvarial cells and the signal transduction mechanisms through which PGE_2 exerts its effects (described above) do not agree with those reported in other cell types. Since it has been suggested that the nature of bone cell responses to various hormones and growth factors may reflect their stage of differentiation or the developmental stage of the

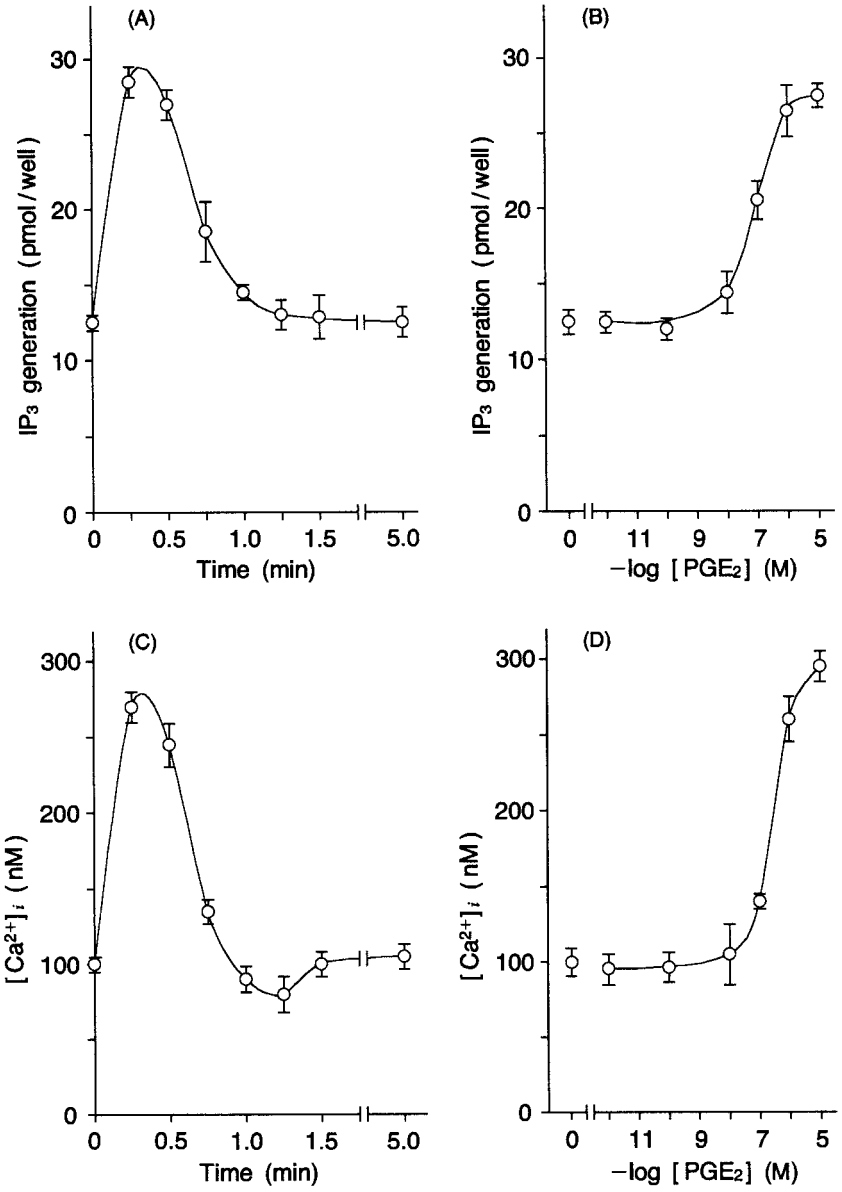

Fig. 6. Time course and dose dependency of the effect of PGE_2 on IP_3 generation and $[Ca^{2+}]_i$ in adult rat calvarial cells. For the determination of IP_3 formation, cells were treated with 10^{-6} M PGE_2 for the indicated times **(A)** or with the indicated concentration of PGE_2 for 15 s **(B)** on day P4. For the determination of $[Ca^{2+}]_i$, the cells were treated with 10^{-6} M PGE_2 for the indicated times **(C)** or treated with the indicated concentrations of PGE_2 for 15 s **(D)** on day P4. The methods used for the determination of $[^3H]IP_3$ and $[Ca^{2+}]_i$ and other conditions were the same as those described under Materials and Methods. Each value represents the mean ±SD of three cultures. The experiment was repeated twice, and the results were essentially the same as those depicted.

tissues from which they are isolated [Aubin et al., 1982; Stein and Lian, 1993; Centrella et al., 1994], it would be interesting to know the effect of PGE_2 on calvarial cells from fetal rats. As shown in Figure 8, PGE_2 at 10^{-6} M stimulated both the area and the number of BN by 90% and 36% over the control values, in the fetal cells, and by 130% and 100%, in the cells from 10-week-old rats, while forskolin significantly decreased these values. The inhibitory effect of forskolin on BN formation was potentiated by the presence of IBMX. By contrast, PGE_2 inhibited the $[^3H]$thymidine incorporation by 32% and 27% in fetal rat calvarial cells and cells from 10-week-old rats, respectively. The results suggest that, although PGE_2 stimulates the differentiation and suppresses the proliferation of calvarial cells from fetal and 10-week-old rats in a manner similar to that demonstrated in adult rats, the degrees of the PGE_2-induced

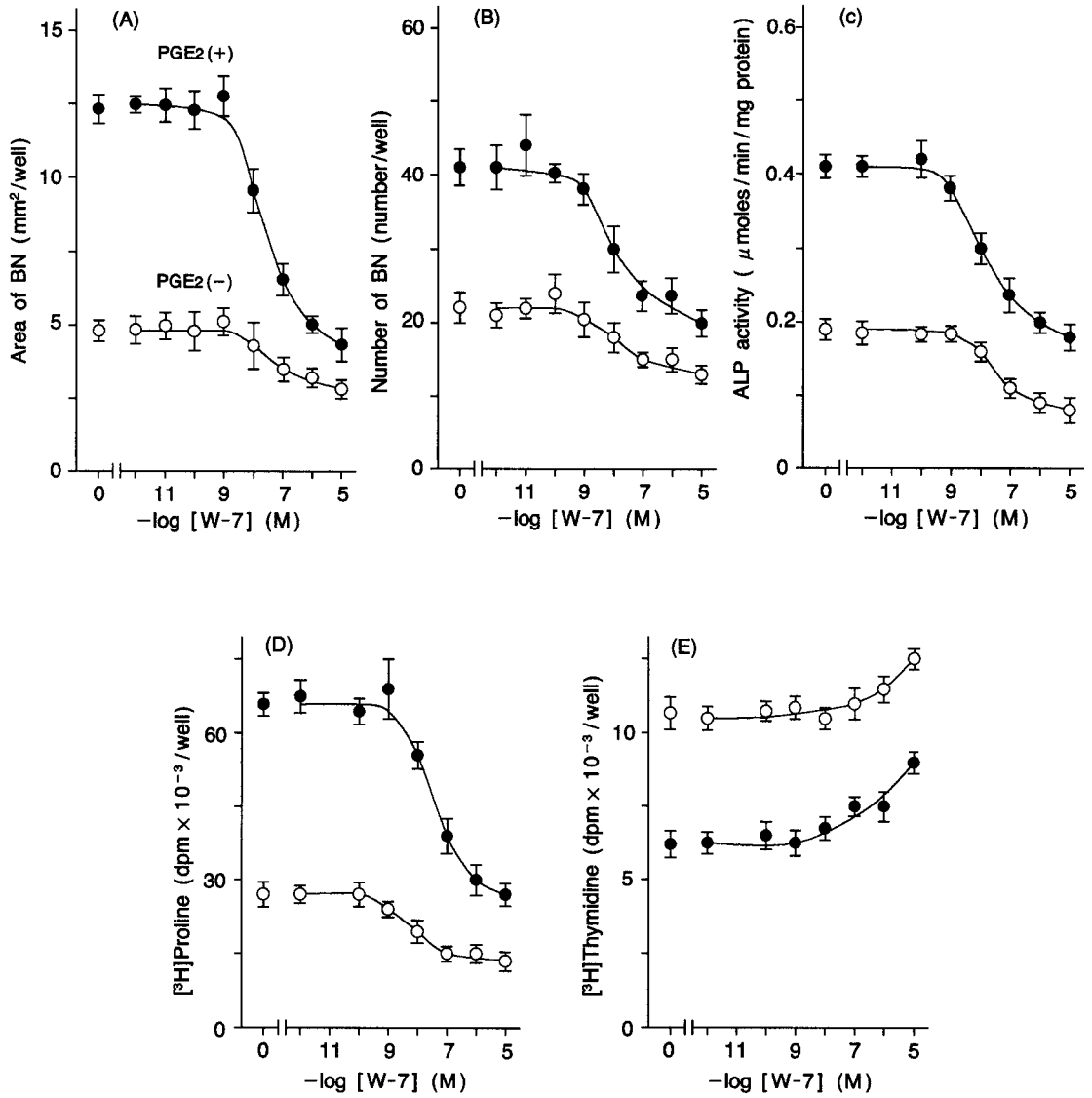

Fig. 7. Effects of W-7 on basal and PGE_2-induced BN formation, ALP activity, collagen synthesis, and DNA synthesis. The cells were treated with indicated concentrations of W-7 in the absence (○) or presence (●) of 10^{-6} M PGE_2 during the last 6 h of day P4. ALP activity **(C)**, [^{3}H]proline incorporation into collagenase-digestible protein **(D)** and [^{3}H]thymidine incorporation into the cells **(E)** were measured at the beginning of day M2. The area **(A)** and number **(B)** of BN were measured on day M18. Other conditions were the same as those described under Materials and Methods. Each point represents the mean ±SD of four cultures. The experiment was repeated twice, and the results were essentially the same as those depicted.

responses are different depending on the cell sources.

It has also been observed in MC3T3-E1 cells that the response to PGE_2 is affected by the timing of the PGE_2 addition to the culture because of the spontaneous differentiation of the cells during the culture period [Suda et al., 1996]. We therefore examined the effects of PGE_2 on BN formation at different time points. In the experiments shown in Figures 1–7, PGE_2 was added on day P4. The cells become confluent on day M3 under the culture conditions employed in this study. As shown in Figure 9, the addition of PGE_2 before confluency (days P3, P4, and M1) significantly stimulated BN formation, with the maximal stimulation obtained on day P4, while significant suppression of BN formation was observed when PGE_2 was added 2 days after confluency (day M5). These results suggest that the responsiveness of adult rat calvarial cells to PGE_2 changes during the culture period.

DISCUSSION

In the present study, using adult rat calvarial cells, we showed that PGE_2 stimulates the BN

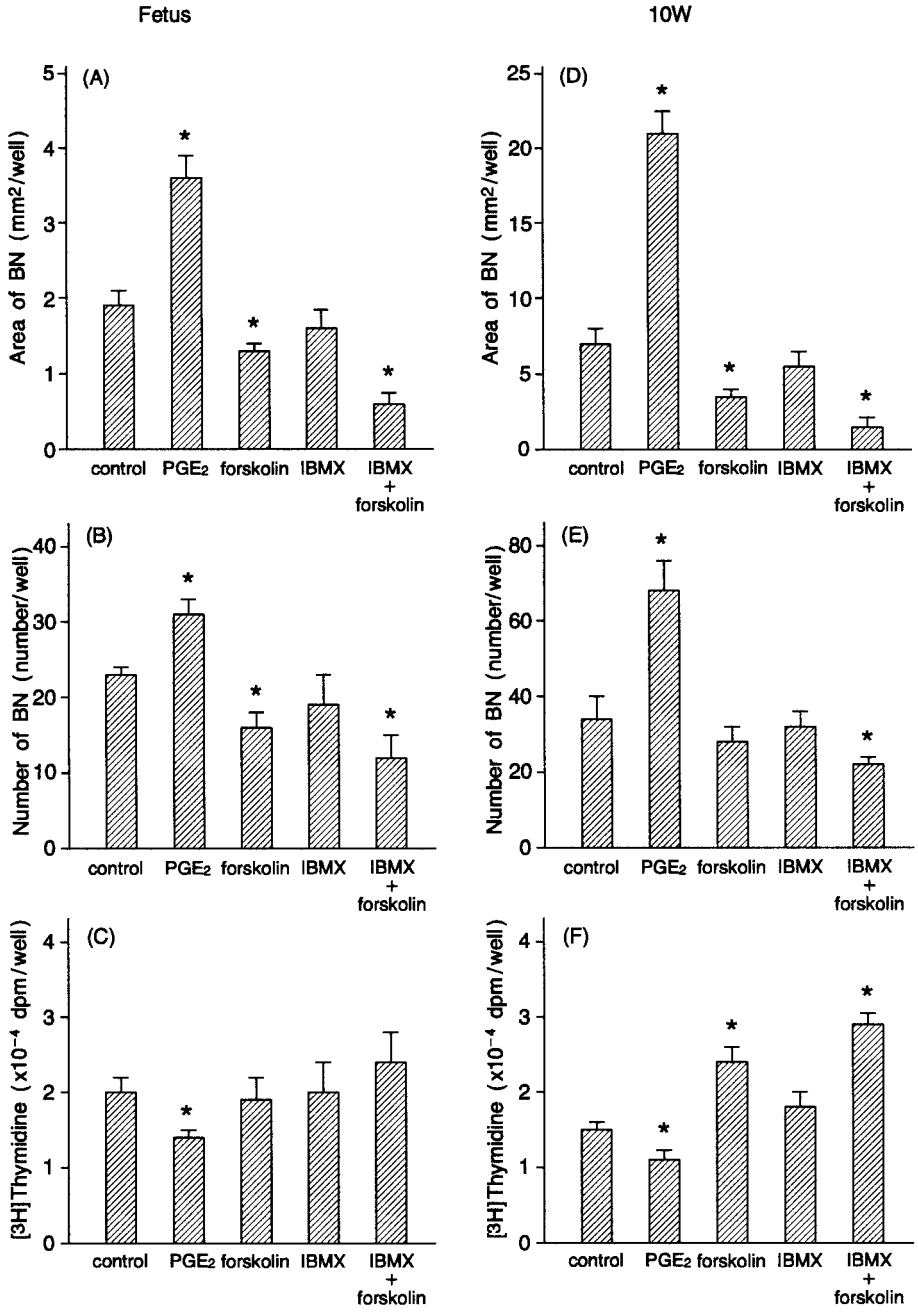

Fig. 8. Effect of PGE_2 on BN formation and DNA synthesis in the cultures of calvarial cells from fetal and 10-week-old rats. The calvarial cells from fetal (A–C) or 10-week-old (D–F) rats were treated with PGE_2 (10^{-6} M), forskolin (10^{-5} M) and/or IBMX (10^{-6} M) during the last 6 h of day P4. The area **(A,D)** and the number **(B,E)** of BN were measured on day M18. [^{3}H]Thymidine incorporation into the cells **(C,F)** was measured at the beginning of day M2. Other conditions were the same as those described under Materials and Methods. Each value represents the mean ±SD of three cultures. The experiment was repeated twice, and the results were essentially the same as those depicted.

formation, ALP activity, and [^{3}H]proline incorporation into collagenase-digestible protein and inhibits DNA synthesis in a dose-dependent manner within the concentration range of 10^{-8}–10^{-5} M. These effects are similar to these observed in MC3T3-E1 cells in the low PGE_2 concentration range. However, neither an inhibition of differentiation nor an enhancement of proliferation was observed at high concentration of PGE_2, indicating that the mode of response of adult rat calvarial cells to PGE_2 is different from that of MC3T3-E1 cells. The biphasic effect of PGE_2 in MC3T3-E1 cells has been explained by the differences in the signal

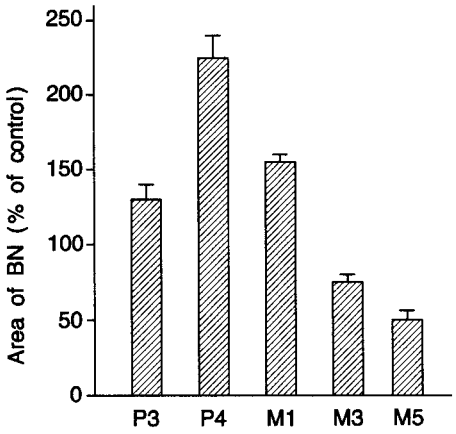

Fig. 9. Effect of varying the time of the addition of PGE_2 on BN formation in adult rat calvarial cells. The cells were treated with 10^{-6} M PGE_2 during the last 6 h of the indicated days. The area of BN was measured on day M18. Other conditions were the same as those described under Materials and Methods. Each value represents the mean ±SD of three cultures. The experiment was repeated twice, and the results were essentially the same as those depicted.

transduction systems used at low and high PGE_2 concentrations. From 10^{-8} M to 10^{-7} M, PGE_2 stimulates adenylate cyclase dose-dependently to elevate the intracellular level of cAMP, which is thought to act as a mediator of cell differentiation [Hakeda et al., 1986, 1987a]. With an approximate EC_{50} value for this effect of around 10^{-7} M, PGE_2 stimulates cell differentiation at low concentrations. At high concentrations, PGE_2 enhances the PI turnover by stimulating PI-PLC, in addition to cAMP generation, resulting in an elevation of $[Ca^{2+}]_i$. PI hydrolysis is thought to mediate the stimulation of proliferation and, at the same time, to counteract the effect of cAMP [Suda et al., 1996]. The approximate EC_{50} value of PGE_2 in the stimulation of PI-PLC has been reported to be approximately 10^{-6} M [Suda et al., 1996].

An interesting finding in the present study was that forskolin suppressed the BN formation, ALP activity, and collagen synthesis dose-dependently and stimulated DNA synthesis in adult rat calvarial cells; these effects were potentiated by the presence of IBMX, indicating that cAMP acts as an inhibitor of differentiation and stimulator of proliferation (Fig. 4). These results do not agree with the observations with MC3T3-E1 cells in which cAMP acts as a stimulator of differentiation [Hakeda et al., 1986, 1987a]. In adult calvarial cells, cAMP concentration rose sharply after PGE_2 treatment but rapidly returned to near the control level. Since the attenuation of cAMP was much more gradual when the cells were treated with forskolin, it is likely that PGE_2 activates phosphodiesterase in addition to adenylate cyclase, resulting in a rapid degradation of newly formed cAMP. Supporting this assumption, the degradation of PGE_2-induced cAMP was suppressed by the addition of W-7, a calmodulin inhibitor. It is therefore unlikely that the adenylate cyclase/cAMP system plays a major role in the PGE_2-induced change in calvarial cell function. In fact, PGE_2 enhanced the cell differentiation and suppressed the proliferation, which was probably caused by the activation of PI-turnover and subsequent increases in $[Ca^{2+}]_i$, since the PGE_2-induced increase in BN formation, ALP activity, and collagen synthesis were abolished in the presence of W-7.

In a variety of cells, a transient increase in $[Ca^{2+}]_i$ upon agonist stimulation is often accompanied by a slow and steady increase in $[Ca^{2+}]_i$ due to the influx of extracellular Ca^{2+} across the plasma membrane. Such a secondary increase in $[Ca^{2+}]_i$ has been observed after PGE_2 stimulation in UMR-106 cells [Yamaguchi et al., 1988]. This was not found in the adult calvarial cells in the present study, indicating that PGE_2 has no effect on the plasma membrane Ca^{2+} channel. The hydrolysis of PIP_2 by PI-PLC results in the production of DAG as well as IP_3, the former being known as an activator of PKC, therefore, DAG may also participate in the signal transduction through the activation of PI-PLC. In MC3T3-E1 cells, both PGE_2 and $PGF_{2\alpha}$ stimulate PI turnover, and the dose dependency of inositol phosphate production is closely related to the elevation of DNA synthesis and reduction in ALP activity [Hakeda et al., 1987b]. It has been suggested that the activation of PKC, but not the increase in $[Ca^{2+}]_i$, mediates the increase in proliferation and decrease in differentiation in MC3T3-E1 cells. This hypothesis is based on the following observations: (1) the elevation of $[Ca^{2+}]_i$ induced by A23187, a Ca^{2+} ionophore, does not cause cell proliferation, and (2) TPA, an activator of PKC, suppresses ALP activity and enhances DNA synthesis. We suggest in the present study that PKC is not involved in the PGE_2-induced functional changes in the adult rat calvarial cells, because neither TPA nor calphostin C affected the PGE_2-induced increases in ALP activity and BN formation. Both the active signal transduction pathway and the

functional changes after PGE_2 treatment seem to be different between the two cell types.

It is unknown why the signal transduction pathways triggered by PGE_2 or the responses that occur subsequent to the activation of each signal transduction pathway differ depending on the cells used. Such a disparity may be attributable to the difference in the differentiation stage of the cells. Signal transduction systems, including the subtype of the PGE_2 receptor, may vary among cells at different differentiation stages. Suda et al. [1996] reported the presence of PGE_2 receptor subtypes (i.e., EP1, EP2, and EP4) in MC3T3-E1 cells and proposed that EP1 promotes cell growth, while EP2 and EP4 mediate the differentiation of osteoblasts. The expressions of the PGE_2 receptor subtypes in other cell types, including adult calvarial cells, remain unknown. The second signals used after the activation of the same receptor subtype could also be different, depending on the differentiation stage. Populations of osteoblastic cell lines are relatively homogeneous, but the differentiation stage may be different from cell line to cell line. Populations of isolated osteoblast-like cells are not homogeneous and are composed of cells at different differentiation stages. Therefore, the net response of the isolated osteoblasts to PGE_2 is the average of the responses of cell populations having PGE_2 receptors and is likely to reflect the properties of the predominant populations which may differ depending on the species and age of the animals and tissues from which the cells are isolated. As for the difference in the age of rats from which these cells are isolated, we examined the effect of PGE_2 in fetal rat calvarial cells and found that this eicosanoid depresses cell proliferation and stimulates their differentiation, similarly to the results obtained with adult cells, indicating that the age of the rats may not be the major reason for the variation of the responses. Centrella et al. [1994] reported that the mode of response to PGE_2 and other PGs was different among cell fractions obtained after five serial collagenase digestions of fetal rat parietal bone. These investigators observed that PGE_2 treatment increased DNA synthesis in the earliest digest, while it inhibited DNA synthesis and collagen synthesis in the mixture of the third to fifth digests. Their results indicate that the method of cell preparation also affects the differentiation stage of the isolated cells.

In the present study, we used adult rat calvarial cells in the last three fractions of five serial enzyme digestions. The cells in these fractions appeared to be more differentiated than those from the first fraction, because of an enrichment with cells exhibiting biochemical characteristics associated with differentiated osteoblasts. We also examined the effect of PGE_2 on the cells from the first fraction. We found that this population does not form BN in the absence or presence of PGE_2 and therefore it was not possible to compare the effect of PGE_2 between the first fraction and the last three fractions. The reason for the lack of ability to form BN in the fist fraction is unknown, but these cells may be less differentiated as compared with the third to fifth fraction. It is also possible that the growth of the cells in the first fraction is hampered by contaminated cells such as fibroblasts, the number of which may be much larger than in the last three fractions. It was recently shown in MC3T3-E1 cells that the effect of PGE_2 is different between cells at confluency and those 5 days after confluency [Suda et al., 1996], suggesting that the mode of response is also affected by the culture period. These differences can be explained by the spontaneous differentiation during the culture period. In most of the experiments in the present study, PGE_2 was added 3 days before confluency (day P4). When PGE_2 was added at confluency (day M3), BN formation was not stimulated and rather was slightly suppressed, and with the addition of PGE_2 2 days after confluency (day M5), BN formation was markedly suppressed. In light of these results, the difference in the time of PGE_2 treatment is at least one of the major causes of the variability of cellular responses due to the cell sources. It is unknown whether the same signal transduction system leads to the different cellular responses depending on the stage of differentiation. Much work will be required to clarify this point.

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