Mechanism Underlying the Aluminum-Induced Stimulation of Bone Nodule Formation by Rat Calvarial Osteoblasts

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(Received September 13, 2003; Accepted September 22, 2003)

The signal transduction mechanism for aluminum (Al^{3+})-induced stimulation of bone formation and its crosstalk with the prostaglandin E_2 (PGE_2) signaling pathway were studied in calvarial osteoblasts from 25-week-old rats (MOB) and those from 90-week-old rats (AOB). Alkaline phosphatase activity, the rate of [^{3}H]proline incorporation into collagenase-digestible proteins, the total area and number of mineralized bone nodules (BN) and the content of calcium in BN, which are the markers for differentiation of osteoblasts, were dose–dependently stimulated by the treatment with Al^{3+} at a concentration range of 10^{-7}–10^{-5} M in the cultures of both MOB and AOB. The stimulatory effects of Al^{3+} on the differentiation markers were abolished by the pretreatment of the cells with pertussis toxin (PTX), an inhibitor of G_i protein, indicating that the effects of Al^{3+} are mediated through a receptor coupled with G_i protein. Al^{3+} increased inositol-1,4,5-triphosphate (IP_3) production and intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) in the cultures of MOB and AOB: these effects were not observed in the presence of PTX, indicating that the effects of Al^{3+} are mediated through the activation of phosphatidylinositol-specific phospholipase C (PI-PLC). We have previously shown that 17-phenyl-ω-trinor-PGE_2, a selective agonist for an EP_1 subtype of PGE_2 receptor (EP_1), stimulates the differentiation markers in the cultures of MOB through the activation of PI-PLC, but not in those of AOB because of the lack of EP_1. The levels of the differentiation markers obtained in the presence of the EP_1 agonist were increased by the addition of Al^{3+} in the cultures of MOB and AOB, while Al^{3+} increased the levels of IP_3 production and $[Ca^{2+}]_i$ in the presence of the EP_1 agonist only in the cultures of AOB. These results indicate a possibility that PI-PLC molecules stimulated by the signal through G_i protein and those stimulated by the signal through EP_1 belong to the same pool and that the Al^{3+} signal through G_i protein induces cell differentiation via a pathway(s) independent of PI-PLC in addition to that (those) dependent on the PI-PLC. We have also shown that 11-deoxy-PGE_1, a selective agonist for an EP_2/EP_4 subtype of PGE_2 receptor (EP_2/EP_4), inhibits cell differentiation in the cultures of both MOB and AOB. Al^{3+} had no effect on the basal levels of cAMP production, but the levels induced by the EP_2/EP_4 agonist were dose–dependently reduced by the treatment with Al^{3+} at a concentration range of 10^{-7}–10^{-5} M. The inhibitory effect of Al^{3+} on adenylyl cyclase was abolished by the pretreatment with PTX. These results indicate that Al^{3+} suppresses adenylyl cyclase activity induced by the EP_2/EP_4-mediated signal through the G_i protein-coupled receptor.

Key words ——— aluminum, osteoblast, bone formation, prostaglandin E_2, signal transduction, G_i protein

INTRODUCTION

Exposure to aluminum (Al^{3+}) has been associated with aplastic bone lesion and osteomalacia characterized by decreased mineralization of osteoid in patients with chronic renal failure undergoing hemodialysis and in those under long-term medication of Al^{3+}-containing phosphate binders.[1–4] However, the mechanism by which Al^{3+} produces the osteodystrophy is controversial. Decreased numbers of osteoblasts and accumulation of Al^{3+} at the mineralization front have been observed in human biopsy samples[5–7] and in bone tissues from experimental animal models,[8] suggesting that Al^{3+} may physicochemically inhibit the formation and growth of hy-

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droxylapatite crystals and/or inhibit mineralization through the action on osteoblasts. The involvement of a relative deficiency of circulating parathyroid hormone has also been suggested in the pathogenesis of Al^{3+}-induced osteomalacia.[6,9]

Conflicting results have also been obtained as to the effect of Al^{3+} on proliferation and differentiation of osteoblasts from in vitro studies.[10–17] Thus, several investigators reported that Al^{3+} decreases the parameters of proliferation and differentiation in the cultures of mouse calvarial cells[10] and clonal osteoblasts-like cell lines such as UMR106-01[11] and ROS 17/2.8,[12] but others reported stimulatory effects of Al^{3+} on these parameters in the cultures of rat[13] and chicken[14] calvarial cells, human normal osteoblasts,[14] and clonal osteoblasts-like cell lines such as TE-85[14–16] and MC3T3-E1.[17] The apparent discrepancy seems to be due to the difference in cells used, culture conditions, concentrations and time of addition of Al^{3+} and other experimental conditions. The purpose of the present study is to clarify the mechanism for changes in the properties of osteoblasts induced by Al^{3+} using the cultures of calvarial cells from young and aged rats.

In our previous report, we proposed a model of signal transduction for the activation of mineralized bone nodule (BN) formation by prostaglandin E_2 (PGE_2), a potent modulator of bone remodeling, in rat calvarial osteoblasts.[18] According to the model, PGE_2 stimulates the production of cAMP, a suppressor of bone formation, by the G_s protein-mediated activation of adenylyl cyclase through an EP_2/EP_4 subtype of PGE_2 receptor (EP_2/EP_4). PGE_2 also activates calmodulin (CaM) by the stimulation of phosphatidylinositol-specific phospholipase C (PI-PLC) and subsequent increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) through an EP_1 subtype of PGE_2 receptor (EP_1). The activated CaM accelerates differentiation of osteoblasts and also stimulates phosphodiesterase to hydrolyze cAMP, resulting in the loss of the EP_2/EP_4-mediated signal. We therefore studied here the effect of Al^{3+} on BN formation in relation to the signaling pathway for PGE_2 in the model described above. We also found in the previous study that EP_1 is lost in osteoblasts from aged rats, by which PGE_2 becomes inhibitory to the bone formation. We therefore used the cultures of calvarial cells isolated from 25-week-old rats (MOB) and those from 90-week-old rats (AOB) to determine the age-related changes in the mechanism of action of Al^{3+} on osteoblasts.

MATERIALS AND METHODS

Cell Culture —— The cells enriched for osteoblasts phenotype were enzymatically isolated from calvariae of 25- and 90-week-old female Wistar rats (CLEA, Tokyo, Japan) according to the method described previously.[18,19] Briefly, frontal and parietal bones from the rats were minced with scissors and digested with a mixture of 0.2% collagenase (Wako Pure Chemical Industries, Tokyo, Japan) and 0.25% trypsin at 37°C for 20 min. The released cells were collected and immediately suspended in F-12 medium (Invitrogen, MD, U.S.A.) supplemented with 15% fetal bovine serum (FBS, Invitrogen). This procedure was repeated further 5 times every 20 min. The released cells from the last four populations, 3–6, were cultured in F-12 medium supplemented with 10% FBS at 37°C. After reaching subconfluence, the cells were collected by a trypsin treatment, plated at a density of 2×10^3 cells/cm^2 in the same medium and cultured for 5 days. At the end of day 5, the medium was changed to α-MEM medium (Invitrogen) supplemented with 10% FBS, 5 mM β-glycerophosphate and ascorbic acid (0.1 mg/ml) and the cells were maintained for a further 23 days.

BN Formation —— At the end of day 28, the cells in 4-well plates were washed three times with phosphate-buffered saline (PBS) and BN were visualized by von Kossa's staining as described previously.[18,19] The total number and area of BN were assessed using a colony counter (BMS-400: Toyo Sokki, Tokyo, Japan). For the determination of calcium content in BN, the cells in 12-well plates were washed three times with PBS, scraped in 1 ml of 1 M HCl, incubated for 24 hr with gentle shaking, and sonicated twice for 10 sec with a sonicator (Ultrasonic Disruptor UD-201: Tomy Seiko, Tokyo, Japan). The calcium content in the sonicate was determined with the use of a commercial kit (Calcium test C Wako: Wako Pure Chemical Industries) based on the 3,3′-bis-[*N*,*N*-bis(carboxymethyl)aminomethyl]-*o*-cresolphthalein method.

Determination of Collagen Synthesis —— The cells in 12-well plates were washed twice with the serum-free α-MEM medium at the beginning of day 7 and incubated for 5 hr with the same medium containing [2,3-^{3}H]proline (1.5 μCi/ml). The cells were washed three times with cold PBS to remove unincorporated radioactivity, lysed by freeze-thawing, extracted in 0.5% Triton X-100, and precipitated with cold 10% trichloroacetic acid (TCA). The acid-insoluble precipitates were collected by centrifugation

at 3000 rpm for 15 min at 4°C and resuspended in 10% TCA to remove free [2,3-^{3}H]proline. This washing procedure was repeated twice, and the amounts of collagenase-digestible proteins and non-collagenous proteins in the precipitates were measured according to the method of Peterkofsky and Diegelmann.[20] Briefly, each acid-insoluble protein was digested by highly purified collagenase (Sigma Chemicals, MO, U.S.A.) for 24 hr at 37°C and the radioactivity in supernatants was counted using a liquid scintillation counter as the rate of [2,3-^{3}H]proline incorporation into collagen.

Assay of Alkaline Phosphatase (ALP) Activity ——— At the beginning of day 7, the cells in 24-well plates were washed three times with PBS, and sonicated twice in 0.3 ml of 50 mM Tris–HCl buffer (pH 7.2) containing 0.5% Triton X-100 and 2 mM $MgCl_2$ for 10 sec at 4°C with the sonicator. The activity of alkaline phosphatase (ALP) in the sonicate was determined according to the method of Lowry *et al.*[21] with *p*-nitrophenylphosphate as a substrate.

Cell Proliferation ——— At the beginning of day 6, the cells in 24-well plates were washed twice with serum-free α-MEM medium and incubated for 3 hr in the same medium containing [methyl-^{3}H]thymidine (1.25 μCi/ml). The cells were washed three times with cold PBS to remove unincorporated radioactivity, followed by two washes of 10% TCA. The cell layers were solubilized in 1 M NaOH, and aliquots of the solubilized cells were diluted into liquid scintillation fluid after neutralization with 1 M HCl, and counted using a liquid scintillation counter as the rate of [methyl-^{3}H]thymidine incorporation into the cells.

Radioimmunoassay (RIA) for PGE_2 ——— At the beginning of day 5, the cells in 24-well plates were incubated with $AlCl_3$ for 24 hr in serum- and Zn^{2+}-free F-12 medium. After the incubation, 0.5 ml of the medium was gently mixed with 0.5 ml of 75% ethanol and 0.01 ml of glacial acetic acid, and applied to an Amprep C_{18} column (100 mg size: Amersham Biosciences, NJ, U.S.A.), which had been primed with 3 ml of 10% ethanol. After washing the column with 3 ml of distilled water and 1.5 ml of hexane, PGE_2 was eluted with 3 ml of ethyl acetate. The eluate was evaporated to dryness under a stream of nitrogen, and reconstituted with 0.1 ml of phosphate buffered gelatin saline (pH 7.0). As it is necessary for radioimmunoassay (RIA) to convert PGE_2 into its methyl oximate derivative, the sample was incubated with methyloxylamine hydrochloride reagent (Amersham Biosciences) at 60°C for 1 hr. The amount of PGE_2 in the sample was determined using a commercial RIA kit (Amersham Biosciences).

Measurement of Inositol-1,4,5-Triphosphate (IP_3) ——— The cells in 12-well plates were cultured in the serum- and Zn^{2+}-free F-12 medium for 24 hr on day 5. During the last 1 hr of the incubation, 10^{-2} M LiCl, an inositolphosphate phosphatase inhibitor, was included in the medium. The cells were treated with the same medium containing various concentrations of $AlCl_3$ for 15 sec, scraped into 1 ml of cold 15% TCA, and incubated for 20 min at 4°C. After centrifugation at 3000 rpm for 15 min at 4°C, the supernatant was treated with water-saturated diethyl ether to remove TCA, neutralized with 0.1 M NaOH, and applied to a wool-plugged Pasteur pipette column containing 2 g of anion exchange resin (AG1-X8 formate: Bio-Lad Laboratories, CA, U.S.A.). Inositol-4-monophosphate, inositol-1,4-diphosphate, and inositol-1,4,5-triphosphate (IP_3) were sequentially eluted from the column by the addition of 8 ml each of 0.1 M formic acid containing 0.2, 0.4 and 1 M ammonium formate, respectively. The amount of IP_3 in the eluted fractions was determined using the use of a commercial RIA kit (Amersham Biosciences).

Measurement of Intracellular cAMP ——— The cells in 12-well plates were cultured in the serum- and Zn^{2+}-free F-12 medium for 24 hr on day 4. During the last 30 min of the incubation, 5×10^{-4} M IBMX, a phosphodiesterase inhibitor, was included in the medium. The cells were treated with the same medium containing various concentrations of $AlCl_3$ for 20 min, scraped into 1 ml of cold 15% TCA, and incubated for 20 min at 4°C. After centrifugation at 3000 rpm for 15 min at 4°C, the supernatant was treated with water-saturated diethyl ether to remove TCA, and neutralized with 0.1 M NaOH. The amount of cAMP was determined using a commercial RIA kit (Yamasa, Chiba, Japan).

Measurement of $[Ca^{2+}]_i$ ——— The cells in 12-well plates were cultured in the serum- and Zn^{2+}-free F-12 medium for 24 hr on day 4. During the last 2 hr of the incubation, 10^{-5} M fra-2/AM, a chelating reagent for Ca^{2+}, was included in the medium to hydrolyze the acetoxymethyl ester fra-2 completely. After the incubation, the cells were treated with various concentrations of $AlCl_3$ for 15 sec and 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 fluorimeter (PerSeptive Biosystems, Tokyo, Japan) according to the method of Grynkiewicz *et al.*[22] as described pre-

Table 1. Sequences of Primers, PCR Conditions and Identity of PCR Products

Gene	Primers ($5' \rightarrow 3'$)	T (°C)	PCR cycle	Product size (bp)	Sequence homology (%)
EP_1	F: AACCAAGAGTGCCTGGGAGG	63	25	410	99
	R: GTTGCAAGAGTCTACTGTGGCG				
EP_2	F: TTCAATGACTCCAGGCGAGT	56	25	540	98
	R: AGGACAGTACTGGACGTACT				
EP_4	F: ATGTCCATCCCCGGAGTCAA	59	25	530	98
	R: AGTCGATGAAGCACCAGGT				
Gq	F: CGATCATGTTTCTGGTAGCGC	59	25	401	98
	R: CAGATTGTACTCCTTCAGGTTCAGC				
Gs	F: ATCATCTTCGTGGTGGCC	59	18	407	99
	R: AGTCGTTGAAGACACGGCG				
GAPDH	F: ACCACAGTCCATGCCATCAC	56	18	452	99
	R: TCCACCACCCTGTTGCTGTA				

Abbreviations are: T, annealing temperature; bp, base pairs; F, forward primer; R, reverse primer.

Table 2. Basal Levels of Differentiation and Proliferation Markers in the Cultures of MOB and AOB

Markers	Osteoblasts	
	MOB	AOB
ALP activity ($\times 10$ μmol/min/mg protein)	1.12 ± 0.081	0.78 ± 0.051*
[^{3}H]prolin incorporation ($\times 10^{-4}$ dpm/well)	1.02 ± 0.052	0.77 ± 0.037*
Area of BN (mm^2/well)	2.32 ± 0.120	1.56 ± 0.082*
Number of BN ($\times 10^{-1}$ colony/well)	2.25 ± 0.192	1.61 ± 0.059*
Calcium content ($\times 10^{-1}$ μg/well)	1.67 ± 0.033	1.01 ± 0.028*
DNA synthesis ($\times 10^{-3}$ dpm/well)	9.12 ± 0.226	5.64 ± 0.154*

Details are described in MATERIALS AND METHODS. Each value represents mean ± S.D. ($n = 4$). *$p < 0.001$ compared with that of MOB ($n = 4$). The experiment was repeated twice and the results were essentially the same as those depicted.

viously.[18,19]

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis ——— At the beginning of day 6, the total RNA of the cells cultured in a 100-mm dish was extracted using TRI ZOL reagent (Invitrogen) based on the acid guanidine thiocyanate and phenol/chloroform method. Aliquots containing 2 μg of total RNA were used for the first-strand cDNA synthesis with M-MLV reverse transcriptase (Wako Pure Chemical Industries) in a final mixture of 20 μl according to the manufacturer's recommendations. The RT was carried out at 37°C for 60 min, and terminated by a 5-min heating at 95°C and subsequent cooling down to 4°C. The RT product for each gene was amplified by PCR using Taq DNA polymerase (Takara, Tokyo, Japan) and a pair of gene-specific primers as shown in Table 1 in a final mixture of 50 μl according to the manufacturer's recommendations. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. PCR started with a 5-min denaturation at 94°C, followed by 18–25 cycles of amplification, extension at 72°C and holding phase at 4°C. We carried out preliminary experiments to determine the number of cycles that provided submaximal amplification of each gene (data not shown). Aliquots of PCR products were run on 2% polyacrylamide gels and stained with SYBR GREEN.

Statistical Analysis ——— Data were analyzed by Student's *t*-test or by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. $p < 0.001$ was considered significant. All data were presented as the means standard deviation of four cultures.

RESULTS

Age-Related Changes in the Effects of Al^{3+} on the Markers for Differentiation and Proliferation of Osteoblasts

Table 2 shows the basal levels of markers for

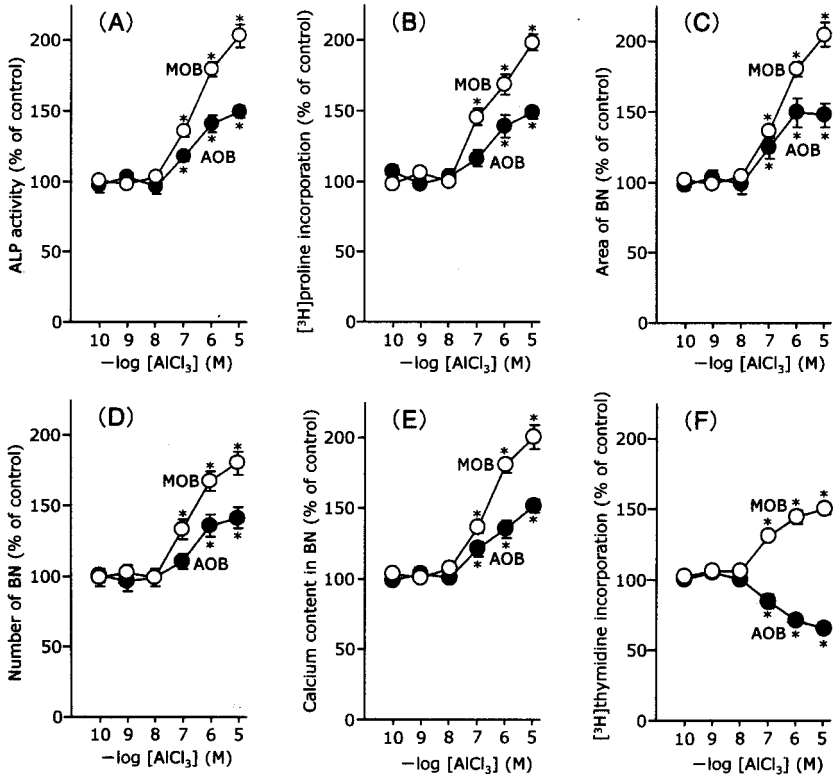

Fig. 1. Effects of Al^{3+} on the Differentiation and Proliferation Markers of Osteoblasts in the Cultures of MOB and AOB

MOB (○) and AOB (●) were treated with various concentrations of Al^{3+} for 24 hr on day 5. The activity of ALP (A) and the rate of [^{3}H]proline incorporation into the collagenase-digestible proteins (B) were measured on day 7. The total area (C) and number (D) of BN and calcium content (E) were measured on day 28. The rate of [^{3}H]thymidine incorporation into the cells (F) was measured on day 6. Each point and vertical bar represents mean value and S.D. ($n = 4$), respectively. Basal levels of all markers were shown in Table 2. The experiment was repeated twice and the results were essentially the same as those depicted. *$p < 0.001$, versus control.

the differentiation and proliferation. The activity of ALP, the rate of [^{3}H]proline incorporation into collagenase-digestible proteins, the total area and number of BN and the content of calcium in BN, which are markers for differentiation, and the rate of [^{3}H]thymidine incorporation into the cells, which is a marker for proliferation, were significantly lower in AOB than in MOB. By the treatment with Al^{3+}, all markers for differentiation increased dose–dependently at a concentration range of 10^{-7}–10^{-5} M in the cultures of both MOB and AOB (Figs. 1A–1E). The maximal values obtained at 10^{-5} M were approximately 2.0 and 1.5-fold greater than control values in the cultures of MOB and AOB, respectively, suggesting the age-dependent decrease in the stimulatory effect of Al^{3+} on the differentiation of osteoblasts. By the treatment with Al^{3+}, the rate of [^{3}H]thymidine incorporation into the cells increased in the cultures of MOB but decreased in those of AOB at a concentration range of 10^{-7}–10^{-5} M (Fig. 1F).

Effect of Al^{3+} on PGE_2 Production by MOB and AOB

Since the functions of osteoblasts can be modified by PGE_2 in an autocrine manner as well as a paracrine manner, Al^{3+} may regulate differentiation and proliferation of MOB and AOB by affecting the rate of PGE_2 secretion from these cells. It is, however, not likely because no significant change was observed in the amount of PGE_2 secreted into the medium after the treatment with various concentrations of Al^{3+} (Fig. 2).

Effect of Al^{3+} on Differentiation and Proliferation of Osteoblasts in the Presence of an EP_1 Agonist

Figure 3 shows the effect of Al^{3+} on the markers for differentiation and proliferation of osteoblasts in the presence of 10^{-6} M 17-phenyl-ω-trinor-PGE_2, a selective EP_1 agonist, in the cultures of MOB and AOB. In the absence of Al^{3+}, the EP_1 agonist increased all markers for differentiation by 70 to 80% and decreased [^{3}H]thymidine incorporation into the cells by approximately 40% in the cultures of MOB,

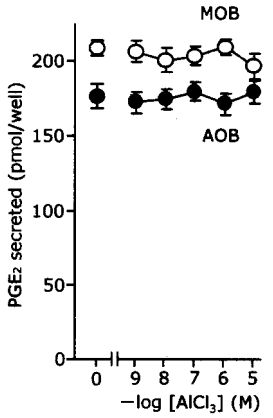

Fig. 2. Effects of Al^{3+} on the Secretion of PGE_2 in the Cultures of MOB and AOB

MOB (○) and AOB (●) were treated with various concentrations of Al^{3+} for 24 hr on day 5, and then the amount of PGE_2 in the medium was determined by RIA. Each point and vertical bar represents mean value and S.D. ($n = 4$), respectively. The experiment was repeated twice and the results were essentially the same as those depicted.

but no changes were observed in those of AOB probably due to the lack of EP_1. In the presence of the EP_1 agonist, Al^{3+} dose–dependently stimulated all differentiation markers in the cultures of both MOB and AOB (Figs. 3A–3E) at a concentration range of 10^{-7}–10^{-5} M, while it was stimulatory and inhibitory to [^{3}H]thymidine incorporation into MOB and into AOB, respectively, at the same concentration range. These results suggest that the effect of Al^{3+} is independent of EP_1. The effects of Al^{3+} were abolished by the pretreatment of the cells with pertissis toxin (PTX), an inhibitor of G_i protein, indicating the involvement of a receptor coupled with G_i protein in the signaling pathway. Figure 4 shows the mRNA expression of PGE_2 receptor subtypes and G proteins in MOB and AOB. The expression levels of EP_1 and G_q protein mRNAs were significantly lower in AOB than in MOB, while no difference was ob-

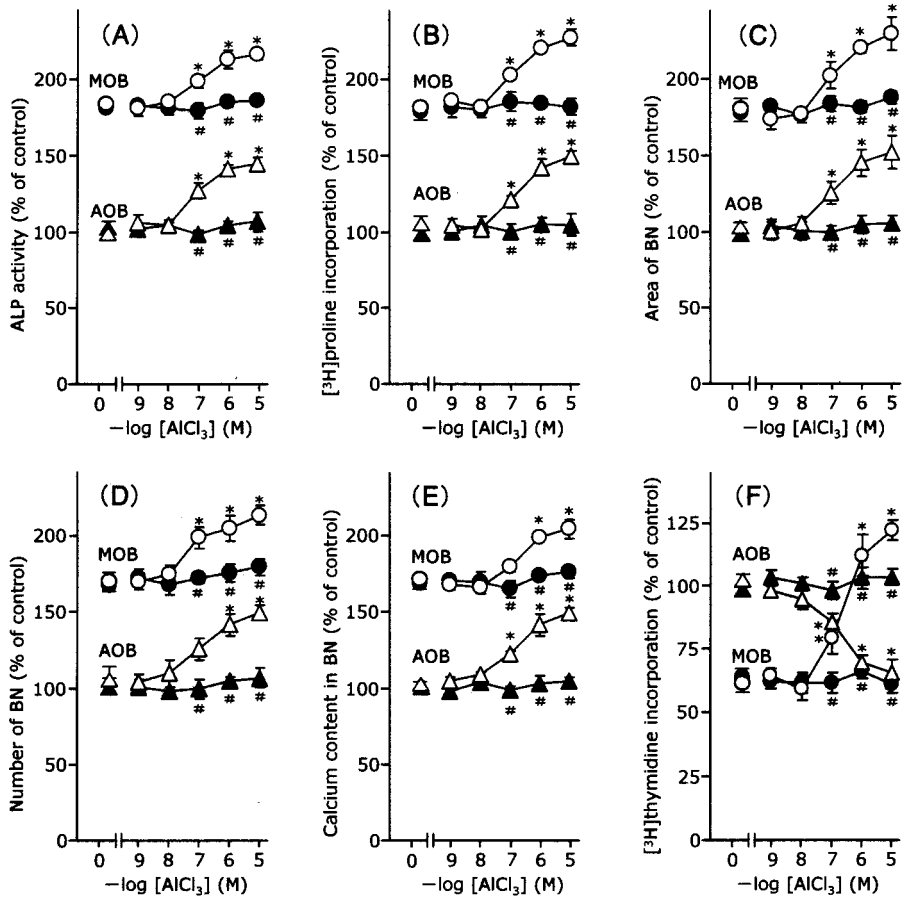

Fig. 3. Effects of Al^{3+} on Differentiation and Proliferation of MOB and AOB in the Presence of EP_1 Agonist

MOB (circle symbols) and AOB (triangle symbols) were pretreated with vehicle (open symbols) or PTX (100 ng/ml, closed symbols) during the last 12 hr of day 4 and then incubated with 10^{-6} M 17-phenyl-ω-trinor-PGE_2 and various concentrations of Al^{3+} for 24 hr. The activity of ALP (A) and the rate of [^{3}H]proline incorporation into the collagenase-digestible proteins (B) were measured on day 7. The total area (C) and number (D) of BN and calcium content (E) were measured on day 28. The rate of [^{3}H]thymidine incorporation into the cells (F) was measured on day 6. Each point and vertical bar represents mean value and S.D. ($n = 4$), respectively. The experiment was repeated twice and the results were essentially the same as those depicted. *$p < 0.001$, versus control. #$p < 0.001$, between vehicle and PTX treatments.

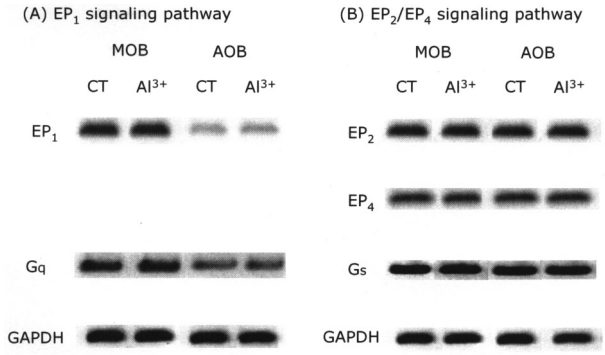

Fig. 4. Effects of Al^{3+} on mRNA Expression Levels of PGE_2 Receptor Subtypes and G Protein Subtypes in the Cultures of MOB and AOB

The cells were treated with vehicle or 10^{-6} M Al^{3+} for 24 hr on day 5. At the beginning of day 6, whole cell RNA was subjected to RT-PCR using specific primers for PGE_2 receptor subtypes (EP_1, EP_2 and EP_4) or G protein subtypes (G_q protein and G_s protein) as described in Materials and Methods. The experiment was repeated twice and the results were essentially the same as those depicted. (A) EP_1 signaling pathway, (B) EP_2/EP_4 signaling pathway. CT, treatment with vehicle; Al^{3+}, treatment with 10^{-6} M Al^{3+}.

served in the expression levels of EP_2, EP_4 and G_s protein mRNAs between MOB and AOB. Treatment with Al^{3+} didn't affect the expression levels of these receptor subtypes and G proteins.

Effect of Al^{3+} on Differentiation and Proliferation of Osteoblasts in the Presence of EP_2/EP_4 Agonist

Figure 5 shows the concentration dependency of the effect of Al^{3+} on markers for differentiation and proliferation of osteoblasts in the presence of 10^{-6} M 11-deoxy-PGE_1, a selective EP_2/EP_4 agonist, in the cultures of MOB and AOB. In the absence of Al^{3+}, the EP_2/EP_4 agonist suppressed the all differentiation markers by 30–50% (Figs. 5A–5E), while it stimulated [^{3}H]thymidine incorporation into the cells by 50% (Fig. 5F). Al^{3+} dose–dependently stimulated all differentiation markers and suppressed [^{3}H]thymidine incorporation into the cells at the concentration range of 10^{-7}–10^{-5} M in the presence of

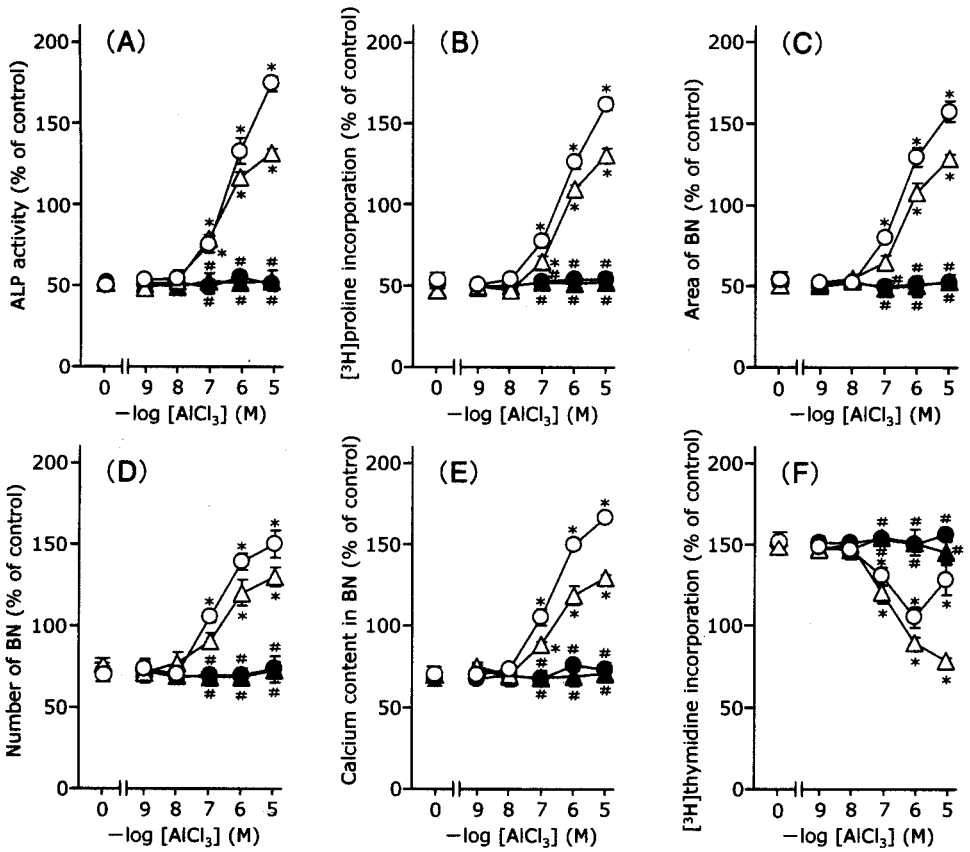

Fig. 5. Effects of Al^{3+} on Differentiation and Proliferation of MOB and AOB in the Presence of EP_2/EP_4 Agonist

MOB (circle symbols) and AOB (triangle symbols) were pretreated with vehicle (open symbols) or PTX (100 ng/ml, closed symbols) during the last 12 hr of day 4, and then incubated with 10^{-6} M 11-deoxy-PGE_1 and various concentrations of Al^{3+} for 24 hr. The activity of ALP (A) and the rate of [^{3}H]proline incorporation into the collagenase-digestible proteins (B) were measured on day 7. The total area (C) and number (D) of BN and calcium content (E) were measured on day 28. The rate of [^{3}H]thymidine incorporation into the cells (F) was measured on day 6. Each point and vertical bar represents mean value and S.D. ($n = 4$), respectively. The experiment was repeated twice and the results were essentially the same as those depicted. *$p < 0.001$, versus control. #$p < 0.001$, between vehicle and PTX treatments.

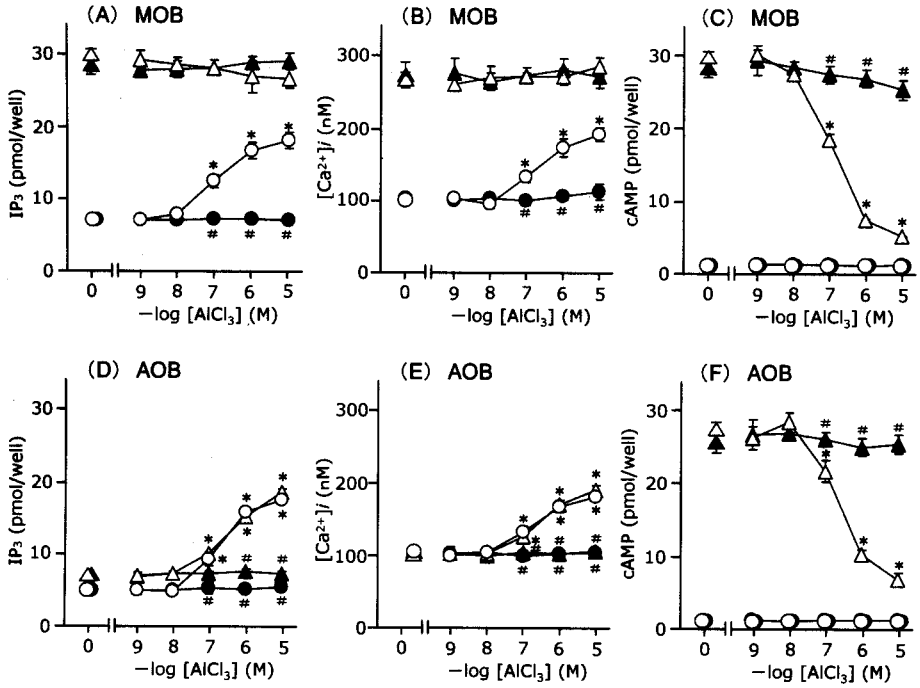

Fig. 6. Effects of Al^{3+} on Intracellular Levels of IP_3 Production, $[Ca^{2+}]_i$, and cAMP Accumulation in the Cultures of MOB and AOB

MOB (A–C) and AOB (D–E) were treated with vehicle (open symbols) or PTX (100 ng/ml, closed symbols) during the last 12 hr of day 4. For the determination of the levels of IP_3 (A, D) and $[Ca^{2+}]_i$ (B, E), the cells were stimulated with various concentrations of Al^{3+} for 15 sec in the presence (triangle symbols) or absence (circle symbols) of 10^{-6} M 17-phenyl-ω-trinor-PGE_2. For the determination of the level of cAMP (C, F), the cells were stimulated with various concentrations of Al^{3+} for 20 min in the presence (triangle symbols) or absence (circle symbols) of 10^{-6} M 11-deoxy-PGE_1. Each point and vertical bar represents mean value and S.D. ($n = 4$), respectively. The experiment was repeated twice and the results were essentially the same as those depicted. *$p < 0.001$, versus control. #$p < 0.001$, between vehicle and PTX treatments.

the EP_2/EP_4 agonist and these effects of Al^{3+} were abolished by the pretreatment of the cells with PTX in the cultures of MOB and AOB (Figs. 5A–5F). These results suggest that Al^{3+} may regulate the EP_2/EP_4 pathway by signaling through the receptor coupled with G_i protein.

Effects of Al^{3+} on the Production of IP_3, $[Ca^{2+}]_i$ and the Accumulation of cAMP

Crosstalk between the signaling pathways for Al^{3+} and PGE_2 was studied by measuring the production of IP_3, $[Ca^{2+}]_i$ and the accumulation of cAMP after the treatment with Al^{3+} (Fig. 6). The treatment of the cells with EP_1 agonist increased the production of IP_3 and $[Ca^{2+}]_i$ in the cultures of MOB (Figs. 6A and 6B), but not in those of AOB (Figs. 6D and 6E). The addition of Al^{3+} to the cultures increased those parameters in the presence and absence of the EP_1 agonist in the cultures of AOB (Figs. 6D and 6E), but only in its absence in those of MOB (Figs. 6A and 6B). The stimulatory effect of Al^{3+} was abolished by the pretreatment of the cells with PTX. These results suggest that the Al^{3+} signal is mediated by the G_i protein-coupled receptor, but not by EP_1, and that the signals through the G_i protein-coupled receptor and those through EP_1 activate the same pool of PI-PLC. The EP_2/EP_4 agonist increased cAMP production in the cultures of both MOB and AOB (Figs. 6C and 6F), and the stimulatory effect was dose–dependently reduced by the addition of Al^{3+} in the absence of PTX, but not in its presence. These results suggest that Al^{3+} suppresses adenylyl cyclase activity induced by the EP_2/EP_4-mediated signal through the G_i protein-coupled receptor.

DISCUSSION

Previous studies concerning the effect of Al^{3+} on osteoblastic functions mainly focused on the mechanism for the development of osteomalacia, since Al^{3+} at high concentrations disturbs normal deposition of calcium phosphate, leading to the incomplete mineralization of bone tissue.[1–9] It has been shown in the present study that the short term treatment of calvarial osteoblasts with Al^{3+} at 10^{-7} M increases the BN formation. Serum levels of Al^{3+} in normal subjects are in the order of 10^{-7} M, while those in

patients with end-stage renal disease undergoing hemodialysis therapy often exceed 5×10^{-6} M.[23,24] It is therefore possible that the physiological level of Al^{3+} serves as a positive regulator of bone formation although *in vivo* experiments in which the circulating level of Al^{3+} is maintained at a low concentration range will be required to prove this assumption.

We have proposed a model for the signal transduction pathway for the PGE_2-induced increase in BN formation in the primary cultures of osteoblasts.[18,19] According to the model, PGE_2 evokes cellular responses of osteoblasts *via* two independent pathways mediated by EP_1 and EP_2/EP_4. Binding of PGE_2 to EP_2/EP_4 leads to the G_s protein-mediated activation of adenylyl cyclase, resulting in the increase in the production of cAMP which is a suppressor of bone formation by osteoblasts. On the other hand, binding of PGE_2 to EP_1 leads to the G_q protein-mediated activation of PI-PLC to produce IP_3, which binds to its receptor on the surface of endoplasmic reticulum to cause the Ca^{2+} release to cytoplasm. Elevation of $[Ca^{2+}]_i$ leads to the activation of CaM, which stimulates the formation of BN by an unknown mechanism. Activated CaM also causes an increase in the activity of phosphodiesterase, resulting in the hydrolysis of cAMP to extinguish the EP_2/EP_4-mediated signal of PGE_2. We propose here a model for the mechanism for induction of BN formation by Al^{3+} and its relationship with the signaling pathways for PGE_2, which is illustrated in Fig. 7. The activation of PI-PLC by Al^{3+} seems to be mediated through a receptor coupled with G_i protein, because the stimulatory effect of the cation is abolished by the pretreatment of the cells with PTX. EP_1 doesn't seem to be involved in the Al^{3+}-induced PI-PLC activation because the cation increased the differentiation markers in AOB, which lacks EP_1. Al^{3+} also seems to regulate the adenylyl cyclase activity induced by a signal through EP_2/EP_4 through the receptor coupled with G_i protein. The identity of the receptor coupled with G_i protein is not known at present. House *et al.*[25] have recently shown that stromal cells, potentially representing osteoblastic functions, expressed the calcium sensing receptor (CaSR), raising a possibility that the receptor is expressed and plays a functional role in the cells of osteoblast lineage. In fact, elevated levels of extracellular Ca^{2+} have several physiologically relevant actions on osteoblasts such as the stimulation of their proliferation and chemotaxis,[26–28] and modulation of the several intracellular second messenger systems.[11,12,29] Although we have identified CaSR mRNA in MOB and AOB by RT-PCR (Kaneki *et al.*, unpublished data), it is not known at present if the receptor is functional in the stimulation of BN formation by Al^{3+}. Further work will be required to clarify this point.

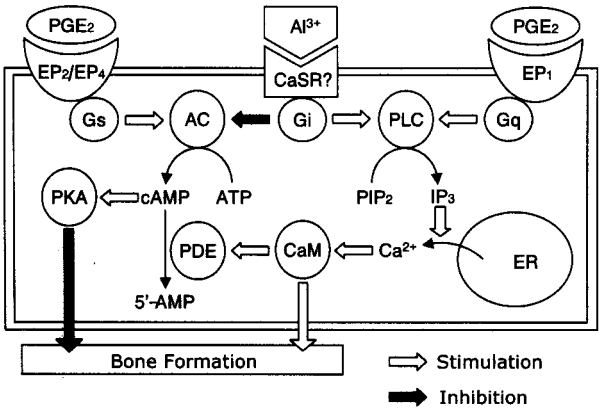

Fig. 7. The Signal Transduction Pathway for Al^{3+}-Induced Cellular Responses in Rat Calvarial Osteoblasts

Binding of PGE_2 or 11-deoxy-PGE_1 to EP_2/EP_4 leads to the activation of adenylyl cyclase (AC) to produce cAMP, which inhibits bone formation through the activation of cAMP-dependent protein kinase (PKA). Binding of PGE_2 or 17-phenyl-ω-trinor-PGE_2 to EP_1 leads to the activation of PI-PLC to produce IP_3, which binds to its receptor on the surface of endoplasmic reticulum (ER), resulting in the release of Ca^{2+}. Ca^{2+} causes the activation of bone formation through the activation of CaM, and also activates a phosphodiesterase (PDE), which suppresses the signaling through EP_2/EP_4 by degrading cAMP. Al^{3+} increases bone formation due to an inhibition of EP_2/EP_4 signaling pathway and an activation of EP_1 signaling pathway through the activation of G_i protein.

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