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Aluminum-Induced DNA Synthesis in Osteoblasts: Mediation by a G-Protein Coupled Cation Sensing Mechanism

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Abstract Aluminum (Al^{3+}) stimulates de novo bone formation in dogs and is a potent stimulus for DNA synthesis in non-transformed osteoblasts in vitro. The recent identification of a G-protein coupled cation-sensing receptor (BoPCaR), which is activated by polyvalent agonists [e.g., gadolinium (Gd^{3+}) > neomycin > calcium (Ca^{2+})], suggests that a similar physiologically important cation sensing receptor may be present in osteoblasts and pharmacologically activated by Al^{3+}. To evaluate that possibility, we assessed whether known BoPCaR agonists stimulate DNA synthesis in MC3T3-E1 osteoblasts and examined the additive effects of Al^{3+} and BoPCaR agonists on DNA synthesis in MC3T3-E1 osteoblast-like cells. We found that Al^{3+}, Gd^{3+}, neomycin, and Ca^{2+} stimulated DNA synthesis in a dose-dependent fashion, achieving 50% effective extracellular concentrations (EC_{50}) of 10 μM, 30 μM, 60 μM, and 2.5 mM, respectively. Al^{3+} displayed non-additive effects on DNA synthesis with the BoPCaR agonists as well as an unrelated G-protein coupled receptor agonist, $PGF_{2\alpha}$, suggesting shared mechanisms of action. In contrast, the receptor tyrosine kinase agonist, IGF-I (10 ηg/ml), displayed additive proliferative effects when combined with $AlCl_3$, indicating distinct signalling pathways. $AlCl_3$ (25 μM) induced DAG levels 2-fold and the phosphorylation of the myristoylated alanine-rich C kinase (MARCKS) substrate 4-fold, but did not increase intracellular calcium concentrations. Down-regulation of PKC by pre-treatment with phorbol 12-myristate 13-acetate as well as PKC inhibition by H-7 and staurosporine blocked Al^{3+}-induced DNA synthesis. Finally, Al^{3+}, Gd^{3+}, neomycin, and Ca^{2+} activated G-proteins in osteoblast membranes as evidenced by increased covalent binding of [^{32}P]-GTP-azidoanilide to putative Gα subunits. Our findings suggest that Al^{3+} stimulates DNA synthesis in osteoblasts through a cation sensing mechanism coupled to G-protein activation and signalling cascades involving DAG and PKC-dependent pathways.

Key words: cation-sensing receptor, BoPCaR, diacylglycerol, gadolinium, fluoroaluiminate, de novo bone formation

Recent data indicate that extracellular cations such as divalent calcium and trivalent aluminum stimulate DNA synthesis in osteoblasts [Kanatani et al., 1991; Quarles et al., 1991]. Indeed, aluminum induces the expression of growth regulating early response genes and cell proliferation in cultures of MC3T3-E1 pre-osteoblasts [Quarles et al., 1991] as well as stimulates DNA synthesis in embryonic chicken calvarial cell and TE85 osteosarcoma cells [Lau et al., 1991]. In addition, the in vivo administration of aluminum chloride stimulates de novo bone formation, a unique process that is independent of bone resorption and generates increments in bone mass [Quarles, et al., 1988, 1990; Galcerin et al., 1987]. These osseous actions of aluminum appear to be derived from activation of osteoblastic precursors [Quarles et al., 1989, 1991]. The mechanism(s), however, whereby aluminum and other cations stimulate osteoblasts to proliferate is unknown.

Extracellular polycations can regulate cellular functions through binding and activation of G-protein coupled membrane receptors. The re-

Abbreviations: BoPCaR, cation-sensing receptor; PLC, phospholipase C; PLD, phospholipid D; $AlCl_3$, aluminum chloride; DAG, diacylglycerol; MARCKS, myristoylated alanine-rich C kinase substrate; PKC, protein kinase C; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PMA, phorbol-12-myristate 13-acetate; H-7, 1-[5-isoquinolinylsulfonyl]2-methylpiperazine; Gd^{3+}, gadolinium; Mg^{2+}, magnesium; HBSS, Hanks balanced salt solution; Me_2SO, dimethyl sulfoxide.

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cent identification in parathyroid cells of a calcium sensing membrane receptor, BoPCaR [Brown et al., 1993] and the discovery that mutations in this receptor underlie abnormal calcium sensing in patients with familial hypocalciuric hypercalcemia [Pollak et al., 1993] provide conclusive evidence for a cation sensing membrane receptor. This cation receptor can be functionally identified by its relative affinity for various cations [gadolinium (Gd^{3+}) > neomycin > calcium (Ca^{2+})] [Brown et al., 1993]. The apparent EC_{50} of BoPCaR for Gd^{3+}, neomycin, and Ca^{2+} is 20 μM, 60 μM, and 3 mM, respectively. Though it is not known if a similar cation receptor is present in osteoblasts, a generalized role for a cation sensor in calcium homeostasis is suggested by BoPCaR expression in calcium regulating tissues such as the kidney and placenta. The possibility that Al^{3+} may activate a BoPCaR-like cation sensor is suggested by the effect of aluminum to mimic calcium-mediated PTH suppression in parathyroid cells [Morrissey et al., 1983]. Thus, it is possible that extracellular Al^{3+} and Ca^{2+} as well as other polyvalent cations might regulate osteoblast function in part though interaction with a similar cation sensing receptor present in the osteoblast membrane.

To examine the possibility of a cation sensing receptor in osteoblasts, we compared in MC3T3-E1 osteoblast-like cells the mitogenic potential of Al^{3+} and known cation-sensing receptor (CaR) agonists, directly evaluated the potential of Al^{3+} to activate G-proteins in osteoblast membranes as assessed by photoaffinity labeling of Gα subunits, and investigated the effects of aluminum on G-protein-linked cellular pathways. We used MC3T3-E1 cells as our model system because early cultures represent nontransformed pre-osteoblasts [Quarles et al., 1992] display a mitogenic response to aluminum [Quarles et al., 1991] and have well-characterized signaling pathways involved in G-protein coupled receptor stimulation of DNA synthesis [Quarles et al., 1993]. We found that a panel of BoPCaR receptor agonists stimulate DNA synthesis in osteoblasts with apparent EC_{50}s characteristic of the reported affinities for the calcium receptor [Brown et al., 1993]. Moreover, aluminum mimics the effects of BoPCaR agonists to stimulate DNA synthesis, activate G-proteins and stimulate G-protein coupled signalling pathways in osteoblasts. Our data suggest that a cation sensing G-protein coupled mechanism is present in osteoblasts that transduces the response to extracellular Al^{3+} as well as polyvalent BoPCaR receptor agonists.

METHODS

Cell Culture

Stock cultures of MC3T3-E1 cells were grown in α-MEM containing 10% (v/v) fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 10% CO_2/90% air at 37°C as previously described [Quarles et al., 1992]. Until the time of study, cells were subcultured every 3–5 days using 0.001% (w/v) pronase to achieve cell detachment. Cultures maintained in this fashion maintain an undifferentiated phenotype [Quarles et al., 1992].

Assay of Mitogenic Activity

We used quiescent (serum-deprived), subconfluent, 3 day old cultures of MC3T3-E1 cells to assess mitogenesis. MC3T3-E1 cells were plated at an initial density of 20,000 cells/ml into 16 mm diameter multi-well plates and pre-incubated for 48 h in α-MEM supplemented with 10% (vol/vol) FBS to permit attachment. Thereafter, quiescence was achieved in subconfluent cultures by removing the medium, washing away the FBS with buffer, and incubating the cells for an additional 24 h in serum-free DMEM/F-12 media containing 0.1% (w/v) bovine albumin (BSA). These media preparations have been previously shown to contain fluoride concentrations less than 0.5 μM [Quarles et al., 1991] and contain a calcium and magnesium concentration of 1.0 and 0.8 mM, respectively.

We compared DNA synthetic rates in control cultures incubated in fresh DMEM/F12 medium containing 0.1% BSA with that of cells treated with the various agonists including aluminum chloride, gadolinium chloride, neomycin, calcium chloride, insulin, insulin-like growth factor I (IGF-I), $PGF_{2\alpha}$, and N^6-monobutyryl cAMP. Additivity experiments were performed by co-administration of these agents with aluminum chloride. Dose-response studies were also performed to establish the maximal effective media concentrations of individual growth factors. When PKC inhibitors were used, they were added 15 min prior to addition of mitogen. In additional studies, cultures were incubated overnight with phorbol-12-myristate 13-acetate (PMA) to down-regulate PKC prior to mitogen addition [Blackshear et al., 1991]. PMA and

thapsigargin were dissolved in Me_2SO at a final media concentration not exceeding 0.1% (v/v), whereas staurosporine was dissolved in absolute alcohol at a final media concentration less than 0.5% (v/v). We assessed DNA synthesis by addition of [^{3}H]-thymidine to the incubation medium at the time of mitogen addition (0.5 μCi/ml, 1 μM) and measured incorporation of [^{3}H]-thymidine into acid precipitable material by modification of previously described techniques [Quarles et al., 1991].

Cellular Uptake of Aluminum

To assess the cellular uptake of aluminum, 50,000 MC3T3-E1 cells were suspended in 5 ml of DMEM/F12 media containing 0.1% (w/v) BSA and incubated at 37°C with 10 μM $AlCl_3$ for up to 6 h. To control for nonspecific binding of this cation, we performed controls that consisted of incubating aluminum with albumin containing media in the absence of cells. After centrifugation, we dissolved the cell pellet by the addition of a 24% nitric acid solution. Aluminum was measured with a flameless atomic absorption spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) according to previously described methods [Quarles et al., 1988]. The cellular uptake of aluminum was calculated by subtracting the total aluminum concentration in the cell pellet from the nonspecific binding.

Measurement of Diacylglycerol (DAG) Kinase

Lipids were extracted by the method of Bligh and Dyer [1959] and total phospholipid phosphate was determined as described [Ames and Dubin, 1960; Van Veldhoven and Manniaerts, 1987]. The total mass of DAG in the lipid extracts was measured using *E. coli* DAG kinase and [γ^{32}P]ATP by previously described methods [Preiss et al., 1986].

Measurement of Cytosolic Calcium Concentration, [Ca^{2+}]i

[Ca^{2+}]i was determined in intact MC3T3-E1 osteoblasts as described previously using the intracellular fluorescent probe Fura2 AM [Quarles et al., 1993]. For these studies, cells grown on glass coverslips were incubated with 20 μM Fura2 AM (Calbiochem, La Jolla, CA) for 1 h in Hanks balanced salt solution (HBSS) containing 0.1% bovine serum albumin, 1.26 mM calcium, and 10 mM Hepes at pH 7.4. Coverslips were placed in a cuvette and continuously perfused with HBSS in the cuvette holder of a Perkin-Elmer LS50 (Norwalk, CT) fluorescence spectrometer. Following agonist addition, real-time measurements were monitored by alternating excitation wavelengths (340 nm/380 nm) every 1.9 s with a microcomputer and deriving data from the ratio of emission at 510 nm. Cytosolic calcium was calculated from a fluorescence maximum in the presence of 6 μM ionomycin and fluorescence minimum in calcium-free HBSS, 5 mM EGTA, pH 8.0, as described by Grynkiewicz et al. [1985].

Assessment of MARCKS Protein Phosphorylation

^{32}P labeling of cells and immunoprecipitation of MARKS protein were carried out as previously described [Quarles et al., 1993; Lobaugh and Blackshear, 1990] using antiserum raised against a conserved sequence from the amino-terminal myristoylated alanine rich C kinase substrate (MARCKS) peptide (a gift from Dr. Perry Blackshear). For these studies, we quiesced and stimulated cells with various agonists in media containing 1% (w/v) BSA. Radioactivity in cellular extracts used for immunoprecipitation was balanced based on trichloroacetic acid-precipitable radioactivity in each sample. ^{32}P-labeled MARCKS protein was separated by SDS-PAGE electrophoresis using a 10% gel, identified by autoradiography and quantified by scanning densitometry (Hoefer Instruments, San Francisco, CA).

Photoaffinity Labeling of G-proteins

[α-^{32}P]-GTP azidoanilide was synthesized as previously described [Offermans et al., 1991a,b], except that the photoaffinity label was purified using thin layer chromatography. Crude membrane protein was obtained from 4 day old MC3T3-E1 that had been quiesced for 24 h by the removal of serum from the incubation media. Photoaffinity labeling of G-proteins was performed using 50 μg of crude membrane protein in the presence and absence of 3 μM of GDP. Membranes were incubated at 37°C for 10 min with 0.6 μCi of [α-^{32}P]-GTP azidoanilide (3160 Ci/mmol) in the presence or absence of 25 μM $AlCl_3$ in 50 μl volumes of 30 mM Hepes, pH 7.5, 100 mM NaCl, 5 mg/l soy trypsin inhibitor with and without 0.3 mM $MgCl_2$. The reaction was stopped by immersion in ice and the membranes were collected by centrifugation at 13,000*g* for 4 min at 4°C, and resuspended in the identical buffer lacking $AlCl_3$ and GDP but supplemented

with 2 mM DTT. After exposure to ultraviolet light for 3 min at 4°C, α-^{32}P-GTP azidoanilide labeled Gα subunits were separated by SDS-PAGE electrophoresis and identified by autoradiography.

Materials

We obtained [^{3}H] thymidine (83 Ci/mmole), [^{32}P]orthophosphoric acid (8,500 Ci/mmole), and [γ^{32}P]ATP and [^{32}P]GTP from Dupont-NEN (Boston, MA). Aluminum chloride ($AlCl_3 \cdot 6H_2O$) was purchased from Fisher (Fairlawn, NJ). Gadolinium chloride ($GdCl_3$) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). All culture materials were obtained from Gibco (Grand Island, NY). Calcium chloride ($CaCl_2$), prostaglandin $F_{2\alpha}$, β-octyl glucoside, sodium ATP, insulin, 1-[5-isoquinolinylsulfonyl]2-methylpiperazine (H-7), phorbol 12-myristate 13-acetate (PMA), staurosporine, and pronase, were obtained from Sigma Chemical Co. (St. Louis, MO). IGF-I was obtained from Ciba-Geigy (Summit, NJ). Thapsigargin was supplied by LC Services Corporation (Woodburn, MA). Bovine albumin Fraction V was obtained from Boehringer Mannheim Corporation (Indianapolis, IN). Sn-1.2-dioleoylglycerol and dioleoylphosphatidylglycerol were purchased from Avanti Polar Lipids (Alabaster, AL). Ionomycin and FURA 2 AM were obtained from Calbiochem (La Jolla, CA). DAG kinase from *Escherichia coli* was generously given by Dr. Yusuf A. Hannun, Department of Medicine, Duke University, Durham, NC.

Statistics

Results are expressed as mean ± SEM. Statistical evaluation for differences between groups was done using one-way analysis of variance [Sokal and Rohlf, 1981]. All computations were performed with the Statgraphics software package (Statistical Graphics Corp, Inc., Princeton, NJ) on a Northgate Elegance SP433 computer.

RESULTS

Effects of BoPCaR Receptor Agonists and Al^{3+} on DNA Synthesis in MC3T3-E1 Osteoblasts

A panel of agonists reported to activate BoPCaR, including gadolinium, neomycin, and calcium, increased DNA synthesis in quiescent MC3T3-E1 osteoblasts in a dose-dependent fashion (Fig. 1A). The apparent half-maximal stimulation of DNA synthesis was approximately 30 μM for Gd^{3+}, 60 μM for neomycin, and 2.5 mM for Ca^{2+}. These concentrations are similar to those required to activate the calcium receptor in parathyroid cells [Brown et al., 1993]. The addition of aluminum to growth-arrested cultures of undifferentiated MC3T3-E1 osteoblasts maintained in serum-free medium also resulted in a dose-dependent stimulation of DNA synthesis (Fig. 1A) [Quarles et al., 1991]. Aluminum maximally stimulated [^{3}H] thymidine incorporation at a media aluminum concentration of 25 μM, achieving rates of DNA synthesis ranging from 3- to 7-fold above quiescent controls in various experiments. Aluminum elicited rates of DNA synthesis in osteoblasts comparable to gadolinium but displayed a lower apparent half-maximal stimulation (Al^{3+} EC_{50} = 10 μM) (Fig. 1A). Both aluminum and gadolinium stimulated DNA synthesis to a greater degree than neomycin and calcium in MC3T3-E1 osteoblasts (Fig. 1A,B).

To evaluate whether aluminum and the BoPCaR agonists share similar mechanisms of action, we performed additivity studies on DNA synthesis between aluminum and these BoPCaR agonists in MC3T3-E1 osteoblasts. Aluminum displayed nonadditive effects on DNA synthesis with all three BoPCaR agonists, suggesting common mechanisms of action (Fig. 1B). Of interest, combinations of Al^{3+} and Ca^{2+} or Al^{3+} and neomycin resulted in rates of DNA synthesis that were less than that of aluminum alone (Fig. 1B). Similar reduction of maximal DNA synthesis induced by Gd^{3+} was obtained with combinations of Gd^{3+} and Ca^{2+} or neomycin (data not shown). Regardless, this mitogenic action of aluminum appears to be due to extracellular aluminum, since MC3T3-E1 cells displayed no demonstrable uptake of aluminum (Fig. 1C).

Aluminum and BoPCaR Agonists Increase [^{32}P]-GTP-Azidoanilide Labeling of Putative G-Proteins in MC3T3-E1 Osteoblasts Membranes

To investigate the possible involvement of G-proteins in aluminum-stimulated DNA synthesis and establish that BoPCaR agonists activate G-proteins in osteoblasts, we assessed covalently labeling of putative G-proteins in osteoblasts membranes with a photoreactive GTP analog, [^{32}P]-GTP-azidoanilide, following agonist stimulation [Offermans et al., 1991a,b]. Minimal but differential binding of [^{32}P]-GTP-

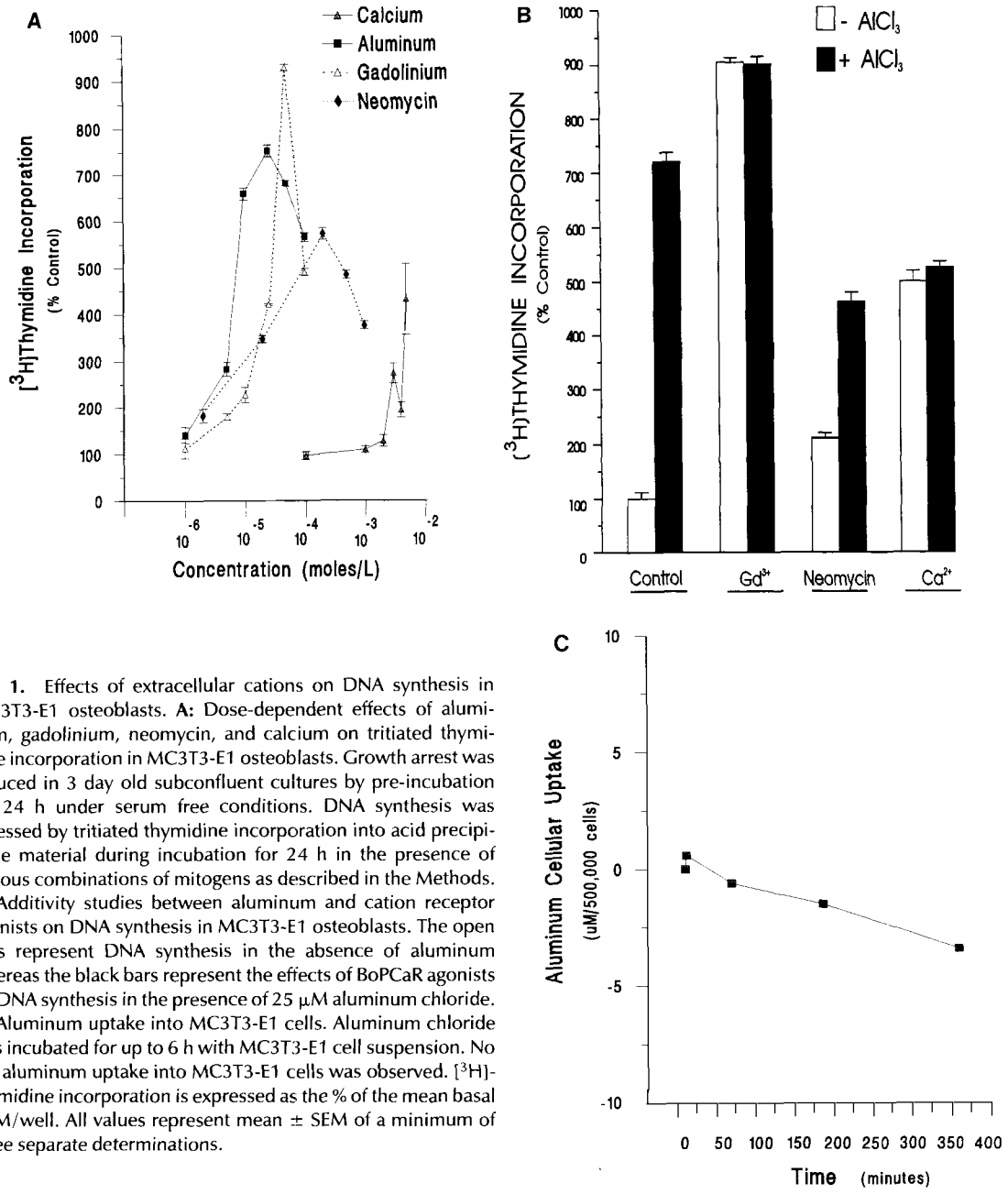

Fig. 1. Effects of extracellular cations on DNA synthesis in MC3T3-E1 osteoblasts. **A:** Dose-dependent effects of aluminum, gadolinium, neomycin, and calcium on tritiated thymidine incorporation in MC3T3-E1 osteoblasts. Growth arrest was induced in 3 day old subconfluent cultures by pre-incubation for 24 h under serum free conditions. DNA synthesis was assessed by tritiated thymidine incorporation into acid precipitable material during incubation for 24 h in the presence of various combinations of mitogens as described in the Methods. **B:** Additivity studies between aluminum and cation receptor agonists on DNA synthesis in MC3T3-E1 osteoblasts. The open bars represent DNA synthesis in the absence of aluminum whereas the black bars represent the effects of BoPCaR agonists on DNA synthesis in the presence of 25 μM aluminum chloride. **C:** Aluminum uptake into MC3T3-E1 cells. Aluminum chloride was incubated for up to 6 h with MC3T3-E1 cell suspension. No net aluminum uptake into MC3T3-E1 cells was observed. [^{3}H]-thymidine incorporation is expressed as the % of the mean basal CPM/well. All values represent mean ± SEM of a minimum of three separate determinations.

azidoanilide to two putative α-subunit G-proteins with apparent molecular weights of 38 and 42 kDa (38 kDa > 42 kDa) was observed in the absence of $AlCl_3$ (Fig. 2A). The addition of $AlCl_3$ (25 μM) increased binding of [^{32}P]-GTP-azidoanilide to both of these proteins, but the effect on the 42 kDa band was most prominent (Fig. 2A). The effect of aluminum to increase [^{32}P]-GTP-azidoanilide binding to the 42 kDa band was observed at concentrations as low as 1 μM (data not shown). The identity of labeled bands as G-proteins was confirmed by blocking studies with GDP. The addition of 3 μM GDP decreased the binding of [^{32}P]-GTP-azidoanilide to all proteins under both basal and agonist stimulated conditions (Fig. 2A).

Similar to aluminum, we found that BoPCaR agonists calcium and neomycin increased [^{32}P]-GTP-azidoanilide labeling of both the 38 and 42 kDa G-protein α-subunits (Fig. 2B). In contrast, gadolinium increased the binding to the 42 kDa but not the 38 kDa protein. In addition, gadolin-

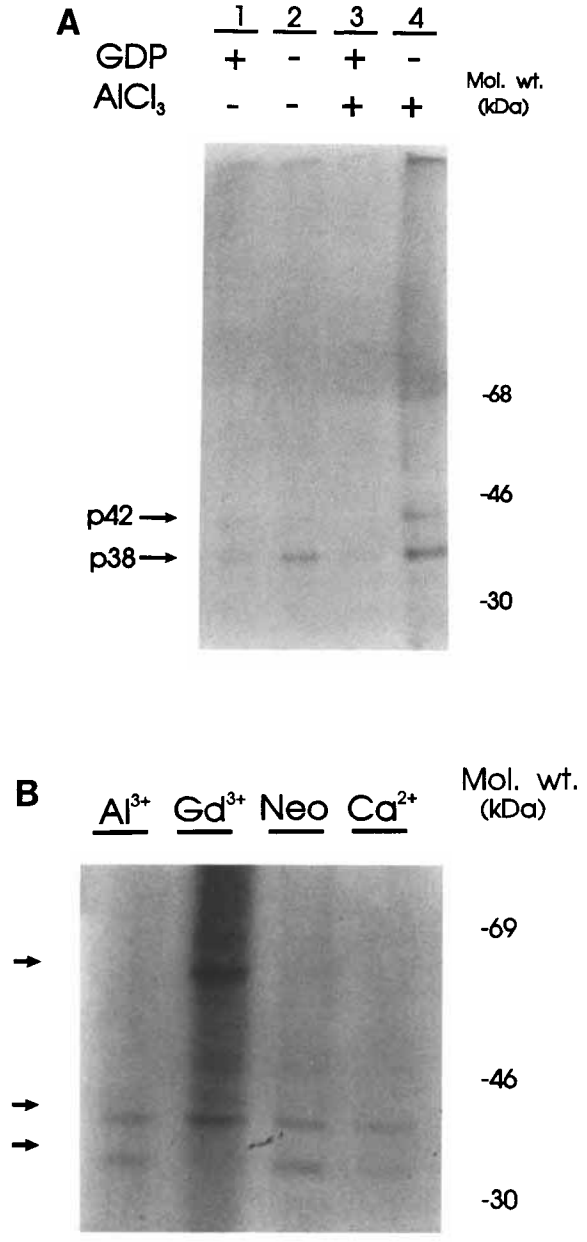

Fig. 2. Photoaffinity labeling of Gα subunits in MC3T3-E1 osteoblasts. **A:** Autoradiograph of aluminum chloride activation of G-proteins in MC3T3-E1 osteoblasts. Membranes were photolabeled with the photoreactive GTP analog, [α-^{32}P]GTP azidoanilide in the presence or absence of 25 μM $AlCl_3$ separated by SDS-PAGE electrophoresis using a 10% gel. Exposure to aluminum increased the incorporation of [α-^{32}P]GTP azidoanilide into 38 and 42 kDa proteins. The aluminum-mediated increase GTP binding to these putative G-proteins was inhibited by 3 μM GDP. Data are representative of three separate experiments. **B:** Autoradiograph comparing photoaffinity labeling of Gα subunits by BoPCaR agonists and aluminum. Membranes were photolabeled with [α-^{32}P]GTP azidoanilide above and separated by SDS-PAGE electrophoresis using a 10% gel. Al^{3+}, neomycin, and Ca^{2+} activated the 38 and 42 kDa proteins, whereas Gd^{3+} induced the labeling of a 42 kDa protein as well as a band that migrated at 68 kDa (upper arrow). Minimal basal activity was observed in the absence of agonists (not shown).

ium increased [^{32}P]-GTP-azidoanilide binding to a protein with an apparent molecular weight of approximately 68 kDa that was not activated by aluminum, calcium, or neomycin stimulation (Fig. 2B).

Comparison of Aluminum Effects on DNA Synthesis With Growth Factors With Known Mechanisms of Actions in MC3T3-E1 Osteoblasts

In separate experiments, we compared the effects of growth factors with known mechanisms of action with that of aluminum in MC3T3-E1 osteoblasts. We have previously shown that the replicative actions of $PGF_{2\alpha}$, a G-protein coupled receptor agonist, and IGF-I, a receptor tyrosine kinase agonist, have distinct mechanisms of action [Quarles et al., 1993]. IGF-I, insulin, and $PGF_{2\alpha}$ increased DNA synthesis in MC3T3-E1 cells in a dose-dependent fashion (Fig. 3A). IGF-1 and $PGF_{2\alpha}$ stimulated maximal [^{3}H] thymidine incorporation at respective doses of 10 and 20 ηg/ml (Fig. 3A). Insulin resulted in maximal rates of DNA synthesis at 10 μg/ml, a dose 1,000-fold greater than that required by IGF-1, (Fig. 3A). Combinations of aluminum with IGF-I resulted in rates of DNA synthesis that were significantly greater than each agonist alone (Fig. 3B), whereas aluminum did not increase the maximal replicative rate achieved in response to $PGF_{2\alpha}$ (Fig. 3B). This pattern of additivity suggests that aluminum and insulin-like growth factors activate distinct pathways, while aluminum and $PGF_{2\alpha}$ share signalling components involved in transducing mitogenic stimuli.

In addition, we tested the effects of N^6-monobutyryl cAMP on DNA synthesis in the presence and absence of aluminum (Fig. 3C). In these experiments, the addition of aluminum chloride (25 μM) to the incubation medium resulted in a 3-fold increase in [^{3}H] thymidine incorporation into DNA compared to control cells; whereas N^6-monobutyryl cAMP antagonized aluminum-stimulated DNA synthesis in quiescent MC3T3-E1 osteoblasts in a dose-dependent fashion. A 50% reduction in the mitogenic actions of aluminum was observed at 0.75 mM N^6-monobutyryl cAMP (Fig. 3C).

The actions of aluminum were not mimicked by complexing aluminum with fluoride to form fluoroaluminate (AlF_4^-), an ubiquitous activator of G-proteins [Rendu et al., 1990]. Fluoroaluminate at concentrations ranging from 1 to 5

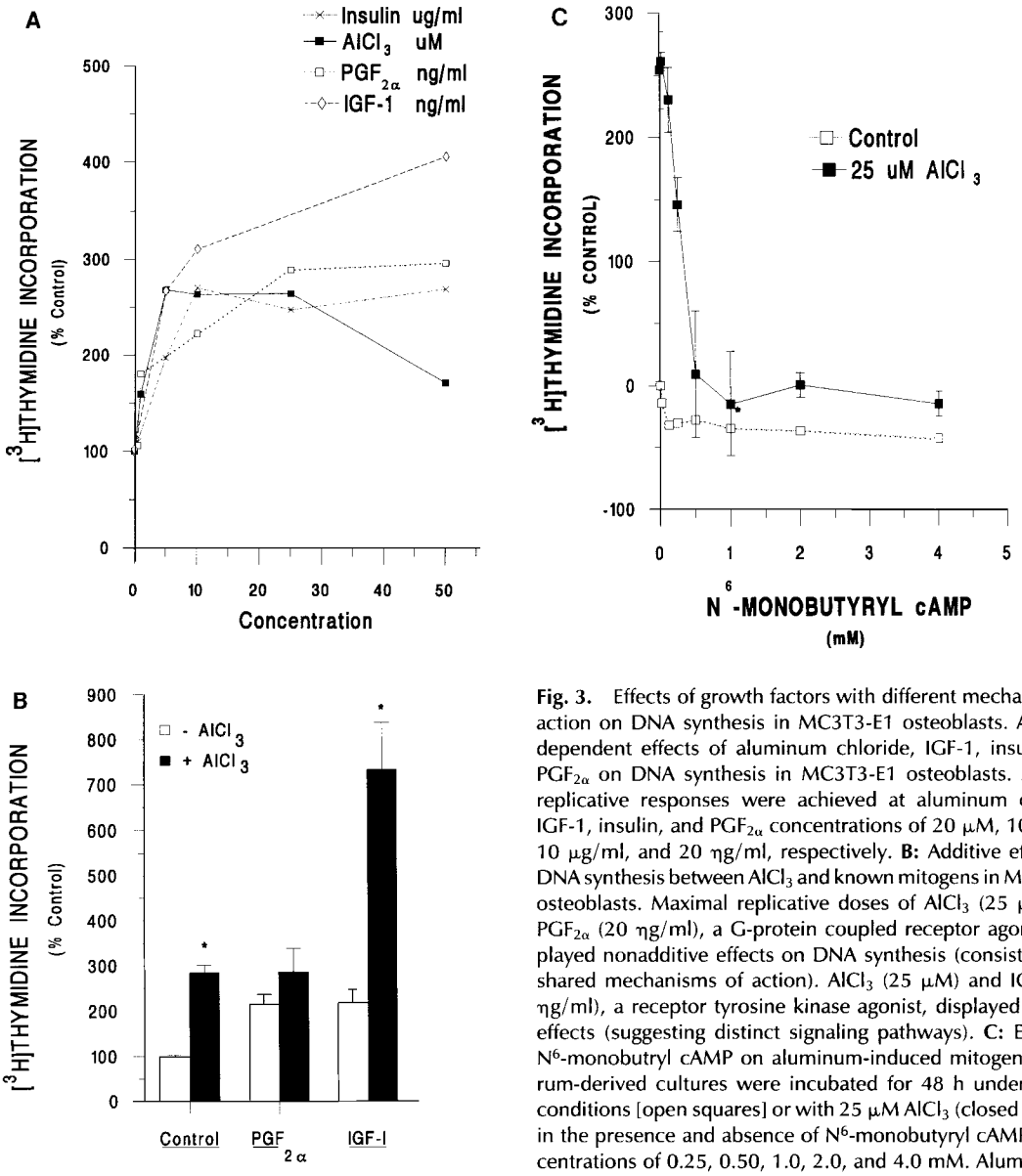

Fig. 3. Effects of growth factors with different mechanisms of action on DNA synthesis in MC3T3-E1 osteoblasts. **A:** Dose-dependent effects of aluminum chloride, IGF-1, insulin, and $PGF_{2\alpha}$ on DNA synthesis in MC3T3-E1 osteoblasts. Maximal replicative responses were achieved at aluminum chloride, IGF-1, insulin, and $PGF_{2\alpha}$ concentrations of 20 μM, 10 ηg/ml, 10 μg/ml, and 20 ηg/ml, respectively. **B:** Additive effects on DNA synthesis between $AlCl_3$ and known mitogens in MC3T3-E1 osteoblasts. Maximal replicative doses of $AlCl_3$ (25 μM) and $PGF_{2\alpha}$ (20 ηg/ml), a G-protein coupled receptor agonist, displayed nonadditive effects on DNA synthesis (consistent with shared mechanisms of action). $AlCl_3$ (25 μM) and IGF-1 (10 ηg/ml), a receptor tyrosine kinase agonist, displayed additive effects (suggesting distinct signaling pathways). **C:** Effects of N^6-monobutryl cAMP on aluminum-induced mitogenesis. Serum-derived cultures were incubated for 48 h under control conditions [open squares] or with 25 μM $AlCl_3$ (closed squares) in the presence and absence of N^6-monobutyryl cAMP at concentrations of 0.25, 0.50, 1.0, 2.0, and 4.0 mM. Aluminum, in the absence of cAMP resulted in a 2.5-fold increase in DNA synthesis. In contrast, the co-administration of cAMP resulted in a dose-dependent inhibition of aluminum-induced [^{3}H] thymidine incorporation, achieving half-maximal suppression at a cAMP concentration of approximately 0.75 mM. Values represent the mean ± SEM expressed as the percent of unstimulated controls. A minimum of three determinations was performed.

μm Al^{3+} and 0.5 to 2.5 mM Fl^- did not stimulate DNA synthesis in these cells. In fact, NaFl alone as well as $AlFl_4^-$ resulted in a dose-dependent decrease in DNA syntheses (data not shown).

Aluminum Effects on Diacylglycerol Levels, Phosphorylation of MARCKS Protein, and Intracellular Calcium in MC3T3-E1 Cells

Next, we examined signalling cascades associated with G-protein receptor activation in osteoblasts. Mitogenic doses of $AlCl_3$ (25 μM) caused a 2-fold increase in DAG levels 5 to 6 min following stimulation of MC3T3-E1 cells. Compared to FBS, aluminum-stimulated DAG production was more rapid in onset and less sustained (Fig. 4A). Aluminum stimulation of DAG was associated with PKC activation as assessed by phosphorylation of the MARCKS protein (Fig. 4B). Treatment of MC3T3-E1 osteoblasts with 25 μM $AlCl_3$ for 10 min resulted in a 4-fold increase

in MARCKS protein phosphorylation above unstimulated controls. Aluminum-induced phosphorylation of MARCKS protein, however, was less than that resulting from a 10 min exposure to 1.6 μM PMA, which was used as a positive control for these studies (Fig. 4B).

In contrast to its effects to increase DAG levels, $AlCl_3$ (25 μM) had no discernible effect to increase intracellular calcium concentrations in MC3T3-E1 osteoblasts (Fig. 5A). The potential of aluminum treated MC3T3-E1 cells to mobilized calcium from intracellular stores, however, was maintained as evidenced by the ability of thapsigargin to induce an immediate and sustained rise in intracellular calcium concentrations following aluminum exposure (Fig. 5B). For comparison with other osteoblast growth factors, we also examined the effects of insulin and $PGF_{2\alpha}$ on intracellular calcium concentrations. We found that insulin, as expected from a receptor tyrosine kinase agonist not coupled to PLC [White, 1991], had no detectible effect on intracellular calcium concentration in MC3T3-E1 cells (Fig. 5B). In contrast, $PGF_{2\alpha}$, which increases intracellular IP_3 through the activation of PLC [Hakeda et al., 1987; Miwa et al., 1990], resulted in an immediate and transient increase in intracellular calcium (Fig. 5B). We have also shown that $PGF_{2\alpha}$ increases MARCKS protein phosphorylation in MC3T3-E1 osteoblasts, consistent with concomitant DAG mediated activation of PKC [Quarles et al., 1993].

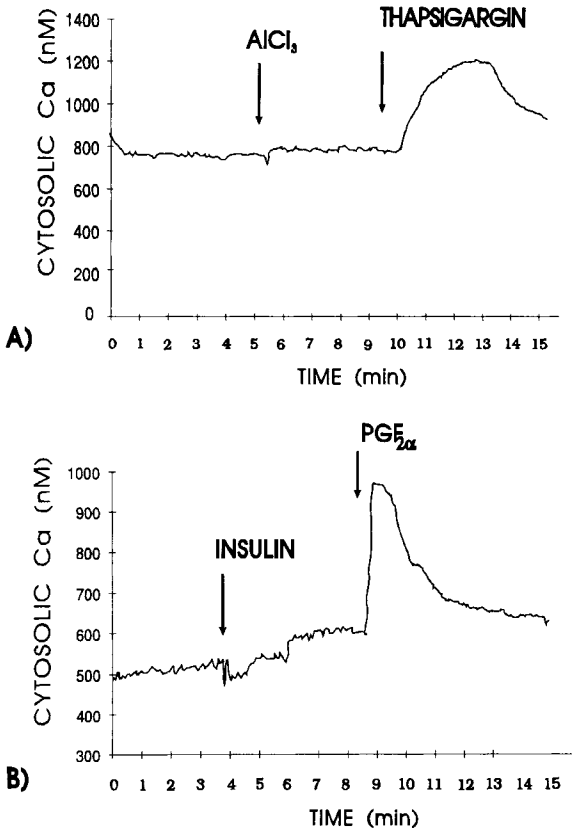

Fig. 4. Effects of aluminum chloride, insulin and $PGF_{2\alpha}$ on cytosolic calcium concentrations. MC3T3-E1 cells were grown onto glass slides and quiesced by incubating the cells for 24 h in the absence of serum. After attaining quiescence, the cells were loaded with Fura 2 for one hour prior to addition of mitogen. Other details are described under Methods. **A:** The addition 25 μM aluminum chloride had no effect on intracellular calcium concentrations, whereas addition of 10^{-7} M thapsigargin (positive control) resulted in a rapid and relatively prolonged increase in intracellular calcium. **B:** Similarly, insulin (10 μg/ml [2 μM]), which is believed to act through a receptor tyrosine kinase that is not coupled to phospholipase C failed to increase intracellular calcium concentrations. In contrast, $PGF_{2\alpha}$ (20 ng/ml [42 nM]) promptly induced a rapid rise in intracellular calcium consistent with it recognized effect to activate the phosphotidyl inositol signal transduction pathway through a G-protein coupled receptor.

Effect of Protein Kinase C Inhibition on Aluminum-Stimulated DNA Synthesis in MC3T3-E1 Cells

We examined the functional role of PKC in aluminum-induced DNA synthesis in MC3T3-E1 osteoblasts. To accomplish this we employed pretreatment with 12-myristate 13-acetate (PMA) to down-regulate PKC as well as chemically distinct agents that are known PKC inhibitors. Overnight pretreatment with 1.6 μM PMA completely inhibited aluminum-induced mitogenesis (Table I). In addition, pretreatment with the PKC inhibitors H-7 or staurosporine also caused significant inhibition of aluminum-stimulated DNA synthesis (Table I).

DISCUSSION

In this study, we present evidence that osteoblasts contain a cation sensing mechanism that converts external signals into proliferative responses. Evidence for a calcium sensing receptor in osteoblasts is derived from the ability of agonists of the recently discovered parathyroid calcium sensing receptor [BoPCaR] [Brown et al., 1993] to stimulate DNA synthesis and activate G-proteins in osteoblasts. Indeed, the BoPCaR agonists gadolinium, neomycin, and calcium stimulated DNA synthesis in MC3T3-E1 osteoblasts at extracellular cation concentrations similar to that required for BoPCaR activation (Fig. 1A). These BoPCaR agonists also stimulated the covalent binding of [^{32}P]-GTP-azidoanilide to putative G-proteins in osteoblast membranes consistent with cation activation of a G-protein

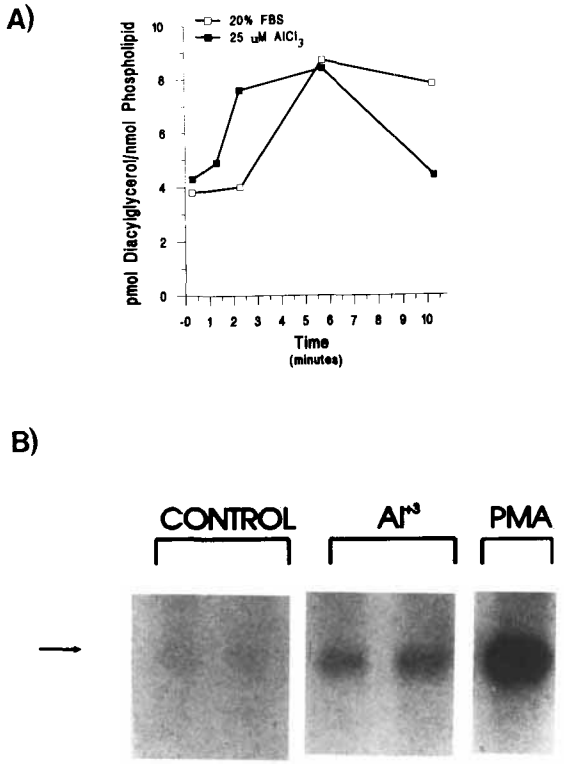

Fig. 5. Effects of aluminum on diacylglycerol (DAG) production and protein kinase C (PKC) activity in MC3T3-E1 osteoblasts. **A:** Time-dependent effects of aluminum chloride ($AlCl_3$) and fetal bovine serum (FBS) on DAG production. DAG was assessed by diacylglycerol kinase assay as described in Methods. $AlCl_3$ (25 μM) resulted in a more rapid but less sustained increase in DAG compared to 20% (V/V) FBS. **B:** $AlCl_3$ effects on PKC as assessed by MARCKS protein phosphorylation. Preconfluent MC3T3-E1 cells were labeled with ^{32}P and then exposed for 10 min to buffer alone (CONTROL), $AlCl_3$ (25 μM), or phorbol-12-myristate 13-acetate (PMA) [1.6 μM]. Phosphorylated MARCKS protein was determined by immunoprecipitation with an anti-MARCKS amino-terminal peptide antiserum and electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel (10% acrylamide Laemmli gel). A representative autoradiograph from two separate studies which shows that aluminum and PMA stimulates phosphorylation of MARCKS protein. Aluminum increased phosphorylation of this PKC substrate by about 20% relative to PMA. Each lane contains a sample derived from a single plate of cells.

coupled receptor. Because cations can bind G-protein coupled receptors as co-factors (such as calcium binding to the calcitonin receptor [Stroop et al., 1993]) and the putative cation sensing receptor in osteoblasts has not been isolated, we can not be certain that our findings are explained by a BoPCaR-like receptor. Nevertheless, our data establish that extracellular divalent and trivalent cations regulate osteoblast proliferation via a cation sensing mechanism.

Our findings provide new insights into the mechanism whereby Al^{3+} stimulates osteoblasts to form bone. We demonstrate that aluminum stimulates DNA synthesis in osteoblasts through activation of a putative G-protein coupled cation sensing system. Several observations support this conclusion. First, the non-additive effects on DNA synthesis between aluminum and BoPCaR agonists is consistent with shared mechanisms of action (Fig. 1A,B). Second, the failure to observe aluminum uptake into osteoblasts suggests that stimulation of DNA synthesis by aluminum requires an extracellular sensing mechanism (Fig. 1C). Third, aluminum behaves like other G-protein receptor agonists in osteoblasts as evidenced by the nonadditive effects on DNA synthesis between aluminum and the unrelated G-protein coupled receptor agonist $PGF_{2\alpha}$. The synergistic effects on DNA synthesis between aluminum and IGF-I, which acts through a receptor tyrosine kinase [White, 1991] not coupled to PLC [Blackshear et al., 1991] also is typical of G-protein coupled receptor agonists. Fourth, the observed increments in DAG (Fig. 4A) and stimulation of PKC (Fig. 4B) in response to aluminum exposure, and the ability of PKC inhibition to block both aluminum and $PGF_{2\alpha}$-induced DNA synthesis (Table I) indicates that aluminum activates intracellular signaling cascades associated with stimulation of G-protein coupled receptors. Finally, similar to other G-protein coupled receptor agonists, mitogenic doses of $AlCl_3$ increased [^{32}P]-GTP-azidoanilide labeling of 38 and 42 kDa Gα subunits in intact osteoblasts (Fig. 2).

Aluminum is known to associate with fluoride to form fluoroaluminate complexes ($AlFl_4^-$) that ubiquitously activate G-proteins (by effectively forming a Gα-GDP-AlF_4^- complex that mimics nonhydrolyzable γGTP activation of Gα subunits [Stadel and Crook, 1989]). Though our study does not exclude the possibility of a direct effect of fluoroaluminate on G-proteins, this appears unlikely for the following reasons. Fluoride contamination of the media is minimal (<0.5 μM) [Quarles et al., 1991] and not sufficient to form $AlFl_4^-$ complexes at concentrations necessary to activate G-proteins (e.g., mM range). Moreover, addition of $AlFl_4^-$ to osteoblasts cultures failed to mimic $AlCl_3$ stimulation

TABLE I. Effects of Protein Kinase C Inhibition on Aluminum-Induced DNA Synthesis in MC3T3-E1 Cells*

Treatment	[^{3}H]thymidine incorporation (cpm/well)		
	Study 1	Study 2	Study 3
Control	8.992 ± 895[a]	9,620 ± 922[a]	7,546 ± 160[a]
+1.6 μM PMA Pre-Rx	7,950 ± 1330[a]	—	—
+25 μM H-7	—	7,723 ± 77[a]	—
+10 pM Staurosporine	—	—	7,637 ± 1,207[a]
25 μM Al^{3+}	36,065 ± 5,244[b]	38,773 ± 1,969[b]	23,594 ± 1,566[b]
+1.6 μM PMA Pre-Rx	9,033 ± 849[a]	—	—
+25 μM H-7	—	22,464 ± 1,899[c]	—
+10 pM Staurosporine	—	—	10,654 ± 1065[a]

*Values are expressed as mean ± SEM of at least three separate determinations. Values sharing a common superscript within a study are not significantly different at $P < 0.05$.

of DNA synthesis but rather caused a dose-dependent inhibition of proliferation. Other investigations have separated the physiologic actions of aluminum from fluoride with regard to DNA synthesis in osteoblasts [Lau et al., 1991] as well as G-protein activation [Miller et al., 1989]. Finally, since fluoroaluminate associates with the inactive GDP-bound form of G-proteins and inhibits the dissociation of GDP from its Gα subunit [Ferguson et al., 1986; De Lean et al., 1980], the resulting Gα-GDP-AlF_4^- complex would likely antagonize rather than increase the photoaffinity binding of [^{32}P]-GTP-azidoanilide to the Gα subunit [Stadel and Crook, 1989]. Thus, the observed increase in [^{32}P]-GTP-azidoanilide labeling of putative G-proteins in response to aluminum is more consistent with receptor mediated activation of G-proteins.

Regardless, our data show that aluminum-induced DNA synthesis is mediated by DAG- and PKC-dependent pathways that are typically activated by G-protein coupled receptor agonists. In this regard, $AlCl_3$ stimulates DAG production without concomitant increases in intracellular calcium (Figs. 4A and 5) and results in PKC activation as measured by MARCKS protein phosphorylation (Fig. 4B). A role for PKC in mediating aluminum actions is directly supported by the ability of protein kinase C inhibition by staurosporine, H-7 [MacLeod et al., 1990] and PMA pre-treatment [Blackshear et al., 1991] to block aluminum-induced DNA synthesis in MC3T3-E1 cells (Table I). Indeed, our findings are consistent with the recognized role of PKC-dependent pathways in mediating proliferation in a variety of cell types [Marx, 1993; Berra et al., 1993], including osteoblasts [Quarles et al., 1992, 1993]. In fact, PKC activation in MC3T3-E1 osteoblasts has recently been shown to stimulate down-stream tyrosine phosphorylation of possible MAP kinases [Quarles et al., 1992], which regulate common early events necessary for initiating DNA synthesis [Marx, 1993].

Our findings do not define the pathway whereby cation-mediated G-protein activation is coupled to DAG production. Increments in DAG independent of intracellular calcium mobilization, however, suggests that Al^{3+} may stimulate phosphotidylcholine breakdown either through PLC and/or PLD mediated hydrolysis of phosphatidylcholine [Billah and Anthes, 1990; Zhang and Abdel-Latif, 1992]. Hydrolysis of phosphatidylcholine is an important step in mitogenic signalling in response to G-protein coupled receptor agonists in a variety of cell types [Exton, 1990; Lorrodera et al., 1990]. Moreover, G-protein(s) that are coupled to PLD activation are pertussis toxin insensitive and display variable responses to fluoride [Qian et al., 1989; Bocckino et al., 1987], similar to our observations regarding aluminum [Fig. 2, Quarles et al., 1991].

The actions of aluminum, however, differ from stimulation of BoPCaR in parathyroid cells and $PGF_{2\alpha}$ receptor activation in osteoblasts. Both BoPCaR and $PGF_{2\alpha}$ receptor activation are associated with PLC-catalysed phophatidylinositol-4,5-bisphosphate hydrolysis with formation of IP_3 and intracellular calcium mobilization [Exton, 1990]. The apparent activation of distinct signalling cascades by aluminum (e.g., disassociation between increments of DAG and intracellular calcium) may occur through activation of different Gα subunits or the differential coupling of G-proteins to various PLC or PLD isoforms that catalyzes phosphatidylcholine hydro-

lysis. Our finding of distinct labeling of Gα subunits by the BoPCaR agonists in osteoblasts (Fig. 2B) suggests variable coupling specificity exists between possible cations sensing receptor isoforms and various members of the G-protein family [Spiegel et al., 1992].

In conclusion, our results provide preliminary evidence for a putative G-protein coupled cation sensing mechanism in osteoblasts that is involved in transducing the mitogenic signal for various polyvalent cations, including aluminum. Though, specific components of the cation sensing system need to be defined (e.g., molecular characterization of the putative G-protein coupled receptor, identification of the Gα subunits, role for phosphatidycholine hydrolysis, characterization of PKC isoforms, and down-stream signalling cascades), our data suggest a model of aluminum-induced DNA-synthesis that includes G-protein activation, genesis of DAG and down-stream stimulation of PKC-dependent pathways. Thus, osteoblasts contain a cation sensing mechanism that may be involved in the physiologic regulation of coupled bone formation as well as mediate the pharmacologic action of aluminum to stimulate de novo bone formation. Unfortunately, the concentrations of aluminum, gadolinium and calcium necessary to activate this putative cation sensor are toxic in vivo. Further investigations to identify additional agents that pharmacologically activate this physiologically relevant polyvalent cation sensing mechanism in osteoblasts may provide a nontoxic means of inducing de novo bone formation in vivo.

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