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Round One
FINAL REPORT

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Round 1 Progress Report: Anabolic Vitamin D Analogs as Countermeasures to Bone Loss

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During the month of June, 1997, the final month of this contract, Dr. Wei Li continued his investigation of the "priming" effect of vitamin D_3 on parathyroid hormone (PTH)-induced elevations in cytosolic free Ca^{2+} in cultured osteoblasts. Unlike the vitamin D steroid, neither estrogen nor progesterone were either to prime the cells' response to PTH. Wei also confirmed previous experimental results suggesting that vitamin D_3 alone does not change cytoplasmic Ca^{2+} levels.

To summarize the progress we made during the period supported by the Round 1 contract, we demonstrated for the first time that vitamin D_3 influences the effect of PTH on bone cell calcium ion levels. This is a rapid effect, taking place within seconds/minutes. This may prove to be a critical contribution to our understanding of bone physiology in that these two hormones are among the most potent regulators of bone calcium content and of systemic calcium homeostasis. Together with the data gathered from the study of astronauts exposed to microgravity for extended periods, these observations suggest the interaction of vitamin D_3 and PTH as a possible therapeutic target in the treatment of bone loss disorders such as osteoporosis and disuse atrophy. Our findings have been accepted for publication (copy attached):

Li, W., **Karin, N.J.** and Farach-Carson, M.C. (1997). 1,25-Dihydroxyvitamin D_3 Enhances Parathyroid Hormone-Induced Increase in Cytosolic Free Calcium in Osteoblastic Cells through a Membrane-Initiated Pathway. Am. J. Physiol. (in press).

Another result from the Round 1 study was our observation that chronic exposure of cultured osteoblasts to vitamin D_3 altered the number of voltage-sensitive Ca^{2+} channels expressed. Estrogen treatment yielded a similar result, suggesting that there is overlap in the mechanism by which these hormones elicit long-term effects on bone cell calcium homeostasis.

$1,25(OH)_2D_3$ enhances PTH-induced Ca^{2+} transients in preosteoblasts by activating L-type Ca^{2+} channels

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Li, Wei, Randall L. Duncan, Norman J. Karin, and Mary C. Farach-Carson. $1,25(OH)_2D_3$ enhances PTH-induced Ca^{2+} transients in preosteoblasts by activating L-type Ca^{2+} channels. *Am. J. Physiol.* 273 (*Endocrinol. Metab.* 36): E599–E605, 1997.—We previously demonstrated electrophysiologically that 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] shifts the activation threshold of L-type Ca^{2+} channels in osteoblasts toward the resting potential and prolongs mean open time. Presently, we used single-cell Ca^{2+} imaging to study the combined effects of $1,25(OH)_2D_3$ and parathyroid hormone (PTH) during generation of Ca^{2+} transients in fura 2-loaded MC3T3-E1 cells. Pretreatment with $1,25(OH)_2D_3$ concentrations, which alone did not produce Ca^{2+} transients, consistently enhanced Ca^{2+} responses to PTH. Enhancement was dose dependent over the range of 1 to 10 nM and was blocked by pretreatment with 5 μM nitrendipine during pretreatment. A $1,25(OH)_2D_3$ analog that activates L-type channels and shifts their activation threshold also enhanced PTH responses. In contrast, an analog devoid of membrane Ca^{2+} effects did not enhance PTH-induced Ca^{2+} transients. The PTH-induced Ca^{2+} transient involved activation of a dihydropyridine-insensitive cation channel that was inhibited by Gd^{3+}. Together, these data suggest that $1,25(OH)_2D_3$ increases osteoblast responsiveness to PTH through rapid modification of L-type Ca^{2+} channel gating properties, whose activation enhances Ca^{2+} entry through other channels such as the PTH-responsive, Gd^{3+}-sensitive cation channel.

calcitropic hormones; vitamin D; bone cells; calcium homeostasis; parathyroid hormone; 1,25-dihydroxyvitamin D_3

BOTH 1,25-DIHYDROXYVITAMIN D_3 [$1,25(OH)_2D_3$] and parathyroid hormone (PTH) play fundamental roles in controlling bone density and systemic Ca^{2+} homeostasis. Bone is a major target tissue for PTH and $1,25(OH)_2D_3$, and cells of the osteoblastic lineage possess receptors for and respond to both hormones (6, 26). The secosteroid $1,25(OH)_2D_3$ activates both genomic and nongenomic (membrane-initiated) pathways in osteoblastic cells, presumably through separate receptor systems (23). The nuclear vitamin D receptor for $1,25(OH)_2D_3$ (nVDR) is well characterized and has been the subject of numerous comprehensive reviews (21). In contrast, the identity of the membrane receptor and its action in controlling intracellular events remain elusive (22). Our laboratory previously demonstrated, using electrophysiological recording techniques, that $1,25(OH)_2D_3$ increases osteoblastic plasma membrane permeability to Ca^{2+} by shifting the threshold of L-type Ca^{2+} channel activation toward the resting potential and prolonging the channel mean open time (4). This phenomenon occurs within milliseconds after addition of $1,25(OH)_2D_3$. These observations led us to hypothesize that $1,25(OH)_2D_3$ exerts a "priming" effect on membrane-initiated Ca^{2+} responses to other calcitropic hormones acting through plasma membrane receptors, such as PTH (13). These responses generally are coupled to Ca^{2+} release from intracellular stores and to nonselective cation channels in the plasma membrane and together produce transient elevations in cytosolic free Ca^{2+} (12). An enhancing effect of $1,25(OH)_2D_3$ would be predicted to augment Ca^{2+} signaling in response to other calcitropic hormones and could be manifested as an increase in either the magnitude or duration of the Ca^{+2} transient.

Synthetic analogs of $1,25(OH)_2D_3$ containing various structural modifications can stimulate subsets of biological activities in target cells. These analogs have been characterized extensively in several laboratories, including our own (reviewed in Ref. 1). Analogs such as 1,24-dihydroxy-22-ene-24-cyclopropyl D_3 (code name BT, also known as calcipotriol) bind well to the nVDR and selectively activate genomic pathways such as those that lead to increased transcription of bone matrix proteins such as osteopontin and osteocalcin (14). Unlike the parent hormone, $1,25(OH)_2D_3$, these analogs produce little or no acute stimulation of Ca^{2+} influx at low nanomolar concentrations (14). Other analogs, in particular those lacking the 1-α-hydroxyl group, such as analogs 25-hydroxy-16-ene-23-yne-D_3 (code name AT) and 25-hydroxy-23-yne-D_3 (code name Y), lack the ability to bind to the nVDR or initiate transcription of matrix proteins but readily increase Ca^{2+} influx into osteoblastic cells (16). These latter analogs also shift the activation threshold for L-type Ca^{2+} channels toward the resting membrane potential (30). We postulated that these readily distinguishable activities reflect a pharmacological distinction between the two receptors for $1,25(OH)_2D_3$, one nuclear and one in or near the plasma membrane (14).

In this study, we used a single-cell Ca^{2+} imaging system to examine the interaction of $1,25(OH)_2D_3$ or two of the previously characterized $1,25(OH)_2D_3$ analogs, AT and BT, with PTH. Specifically, we examined the potential Ca^{2+}-enhancing effect of the secosteroids with regard to increases in the concentration of free intracellular Ca^{2+} ($[Ca^{2+}]_i$) induced by PTH in preosteoblastic MC3T3-E1 cells loaded with fura 2. A nonfusing, premyocytic cell line, BC_3H_1, which expresses $1,25(OH)_2D_3$-responsive L-type plasma membrane Ca^{2+} channels at high levels characteristic of excitable tissues (3, 10), was studied for comparative purposes. The role of Ca^{2+} influx through voltage-sensitive Ca^{2+} channels present in the plasma membrane in the

1,25(OH)$_2$D$_3$-enhancement phenomenon was demonstrated using inhibitors of channel function. Interaction with the Gd^{3+}-inhibitable, mechanosensitive cation channel found in osteoblasts (12) was also revealed for the first time.

MATERIALS AND METHODS

Materials. Coverslip tissue culture dishes were obtained from MatTek (Ashland, MA). Fura 2-AM, the acetoxymethyl ester of the Ca^{2+}-sensitive fluorescent dye, fura 2, was purchased from Molecular Probes (Eugene, OR). Thapsigargin was obtained from Calbiochem (La Jolla, CA). Bovine PTH-(1—34), nitrendipine, Gd^{3+}, and other chemicals were purchased from Sigma Chemical (St. Louis, MO). 1,25(OH)$_2$D$_3$ was from Biomol Research Laboratories (Plymouth Meeting, PA), and structural analogs were kindly provided by Dr. Anthony Norman (University of California at Riverside, Riverside, CA).

Cell culture. MC3T3-E1 cells, a preosteoblastic line derived from neonatal mouse calvarial bone, were provided by Dr. Renny Franceschi and maintained as culture stocks in ascorbate-free medium containing 10% fetal bovine serum, as described previously (15). Growth phase BC_3H_1 premyocytes were cultured in Dulbecco's modified Eagle's medium (DMEM)-Ham's F12 (1:1) medium containing 10% fetal bovine serum as described (25). Cells were plated onto coverslip dishes in DMEM containing 10% fetal bovine serum 2 days before the day of the experiment. All cells were subconfluent at the time of the experiments.

Intracellular Ca^{2+} measurements. We used a single-cell Ca^{2+} imaging system (Intracellular Imaging, Cincinnati, OH) to perform intracellular Ca^{2+} measurements (28). After the medium was removed from the dishes, cells were rinsed with Hanks' balanced salt solution (HBSS) (140 mM NaCl, 4.2 mM KCl, 0.5 mM NaH_2PO_4, 0.4 mM Na_2HPO_4, 0.4 mM $MgSO_4$, 0.3 mM $MgCl_2$, 1 mM $CaCl_2$, 6 mM glucose, 0.1% bovine serum albumin, and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4) and then loaded with 3 μM fura 2-AM in HBSS for 30 min at 37°C. The conditions were chosen to avoid probe compartmentalization and to maximize cytoplasmic dye localization. The loaded cells were incubated further for 15 min with HBSS alone to allow the complete deesterification of fluorescent probe. Fura 2 fluorescence was visualized with a Nikon inverted microscope using a Nikon ×40 fluor objective. The cells were illuminated with a xenon lamp equipped with quartz collector lenses. A shutter and filter changer containing the two different interference filters (340 and 380 nm) were computer controlled. Emitted light was passed through a 430-nm dichroic mirror, filtered at 510 nm, and imaged with an integrating charge-coupled device video camera. Four to eight cells were measured within each field. Consecutive frames obtained at 340- and 380-nm excitation were compared as a ratio (F_{340}/F_{380}), and $[Ca^{2+}]_i$ in each cell was calculated from F_{340}/F_{380} by comparison with fura 2 free acid standards. Individual Ca^{2+} traces shown in Figs. 1–4 are computer-generated population means derived from simultaneous recording of $[Ca^{2+}]_i$ in the four to eight single cells in a microscopic field. Each experiment was repeated at least three times, and Figs. 1–4 were constructed from representative experiments.

1,25(OH)$_2$D$_3$ and structural analogs. 1,25(OH)$_2$D$_3$ and structural analogs AT and BT were stored as stock solutions in absolute ethanol in the dark at −20°C until use. The structural integrity and concentrations of the compounds were routinely monitored from the absorption spectra and by comparison of the absorbency ratio at 264/228 nm, as described previously (4). Solutions with a ratio <1.6 were discarded.

Other methods. Bovine PTH-(1—34) was dissolved in distilled water. Thapsigargin and nitrendipine were maintained and dispensed from stock solutions in dimethyl sulfoxide (DMSO) or absolute ethanol, respectively. All reagents were stored in the dark at −20°C. The delivery vehicle was used as the control in all experiments.

RESULTS

1,25(OH)$_2$D$_3$ enhances PTH-induced increases in cytosolic Ca^{2+} concentration in preosteoblastic MC3T3-E1 cells. In our initial experiments, we measured the ability of nanomolar concentrations of 1,25(OH)$_2$D$_3$ to induce transient rises in $[Ca^{2+}]_i$ in single cells representing the preosteoblastic and premyocytic phenotypes, both previously shown to express L-type Ca^{2+} channels responsive to secosteroids (4, 10, 20). As shown in Fig. 1, exogenously added 1,25(OH)$_2$D$_3$ (10 nM) produced an immediate and rapid increase of $[Ca^{2+}]_i$ in fura-loaded premyocytic BC_3H_1 cells (Fig. 1*A*) but no significant $[Ca^{2+}]_i$ increase in preosteoblastic MC3T3-E1 cells (Fig.

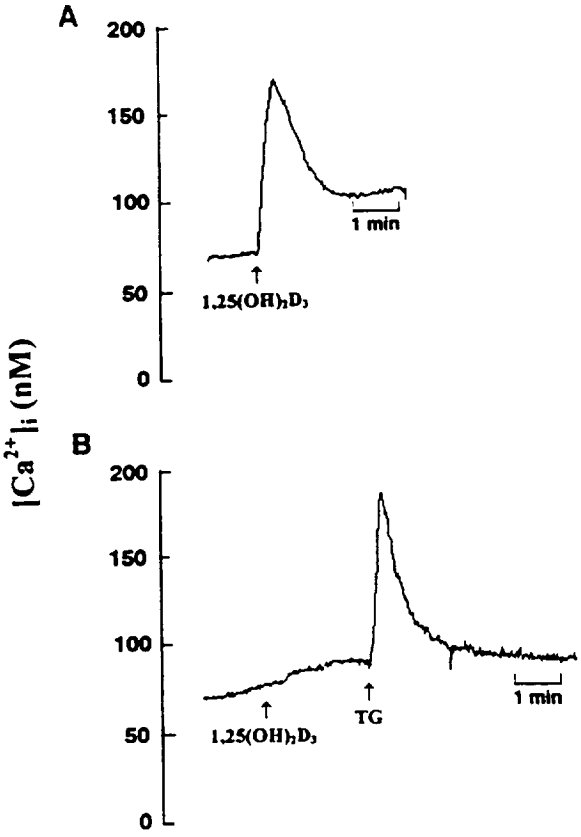

Fig. 1. Effect of 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] on concentration of free intracellular Ca^{2+} ($[Ca^{2+}]_i$) in growth phase myocytic BC_3H_1 cells and preosteoblastic MC3T3-E1 cells. Measurements of $[Ca^{2+}]_i$ were made using a single-cell Ca^{2+} imaging system, as described in MATERIALS AND METHODS. *A*: BC_3H_1 cells treated with 10 nM 1,25(OH)$_2$D$_3$ demonstrate a Ca^{2+} transient that peaks within 15 s and then rests at new baseline. *B*: MC3T3-E1 cells treated with same concentration of 1,25(OH)$_2$D$_3$ do not show a similar Ca^{2+} transient but will release Ca^{2+} from intracellular stores in response to 5 μM thapsigargin (TG). First arrow denotes time of addition of 1,25(OH)$_2$D$_3$.

1*B*). The lack of response in MC3T3-E1 cells is not attributable to an absence of releasable Ca^{2+} in intracellular stores, because addition of thapsigargin immediately produced a Ca^{2+} transient (Fig. 1*B*). Depolarization of the MC3T3-E1 cells with 60–120 mM extracellular K^+ also failed to elicit a Ca^{2+} signal detected by fura 2, although $^{45}Ca^{2+}$ influx studies showed that depolarization triggered an increase in Ca^{2+} uptake within 2 min (data not shown). We next examined the potential ability of $1,25(OH)_2D_3$ to enhance PTH effects on $[Ca^{2+}]_i$ in MC3T3-E1 preosteoblasts, a direct test of our hypothesis that the left shift in activation potential toward the resting potential would augment development of the Ca^{2+} transient induced by PTH. The traces presented in Fig. 2 show that in MC3T3-E1 cells, pretreatment with 10 nM $1,25(OH)_2D_3$ for 10 min before PTH stimulation (Fig. 2*A*) enhanced the PTH-induced Ca^{2+} transient compared with control pretreatment with vehicle (ethanol) alone (Fig. 2*B*). To investigate if influx through L-type Ca^{2+} channels is required for the enhancement effect, we tested whether inclusion of 5 μM nitrendipine, a dihydropyridine blocker of L-type Ca^{2+} channels, would attenuate the enhancement effect of $1,25(OH)_2D_3$. As seen in Fig. 2*C*, the Ca^{2+} transient induced by PTH after treatment with both $1,25(OH)_2D_3$ and nitrendipine was comparable to that produced by PTH in cells treated with vehicle alone (compare Fig. 2, *B* and *C*). Because nitrendipine did not block the PTH-induced Ca^{2+} transient (Fig. 2*C*), we tested whether this transient could be blocked by Gd^{3+}, a lanthanide cation that inhibits stretch-activated Ca^{2+}-conducting channels known to be present in osteoblasts (12). As shown in Fig. 2*D*, addition of 10 μM Gd^{3+} completely abolished the PTH-induced Ca^{2+} transient, even after addition of $1,25(OH)_2D_3$. The same effect was seen if the Gd^{3+} was added during the pretreatment period (data not shown). Even when intracellular stores were full (Fig. 1*B*), the elimination of extracellular Ca^{2+} completely abolished development of the Ca^{2+} transient after addition of $1,25(OH)_2D_3$ and PTH, indicating that influx of extracellular Ca^{2+} was required for development of the Ca^{2+} signal in response to PTH (data not shown). The increased slope after addition of PTH was a common, but not invariable, occurrence during repetition of these experiments. At present we have no explanation for this phenomenon. In further studies, we tested whether the enhancing effect on PTH-induced influx through Gd^{3+}-sensitive channels produced by $1,25(OH)_2D_3$ was dose dependent. An enhancement of the PTH-induced increase in $[Ca^{2+}]_i$ by pretreatment with 5 nM $1,25(OH)_2D_3$ was seen, although the magnitude of $[Ca^{2+}]_i$ rise was less than that induced by pretreatment with the 10-nM dosage (compare Fig. 3, *A* and *B*). Treatment with 1 nM $1,25(OH)_2D_3$ produced a barely detectable enhancement of PTH-induced Ca^{2+} signals (Fig. 3*C*). Simultaneous addition of $1,25(OH)_2D_3$ and PTH did not increase the magnitude or duration of the Ca^{2+} transient (data not shown) relative to that induced by PTH alone.

BC_3H_1 premyocytes did not exhibit Ca^{2+} transients in response to the addition of PTH (data not shown),

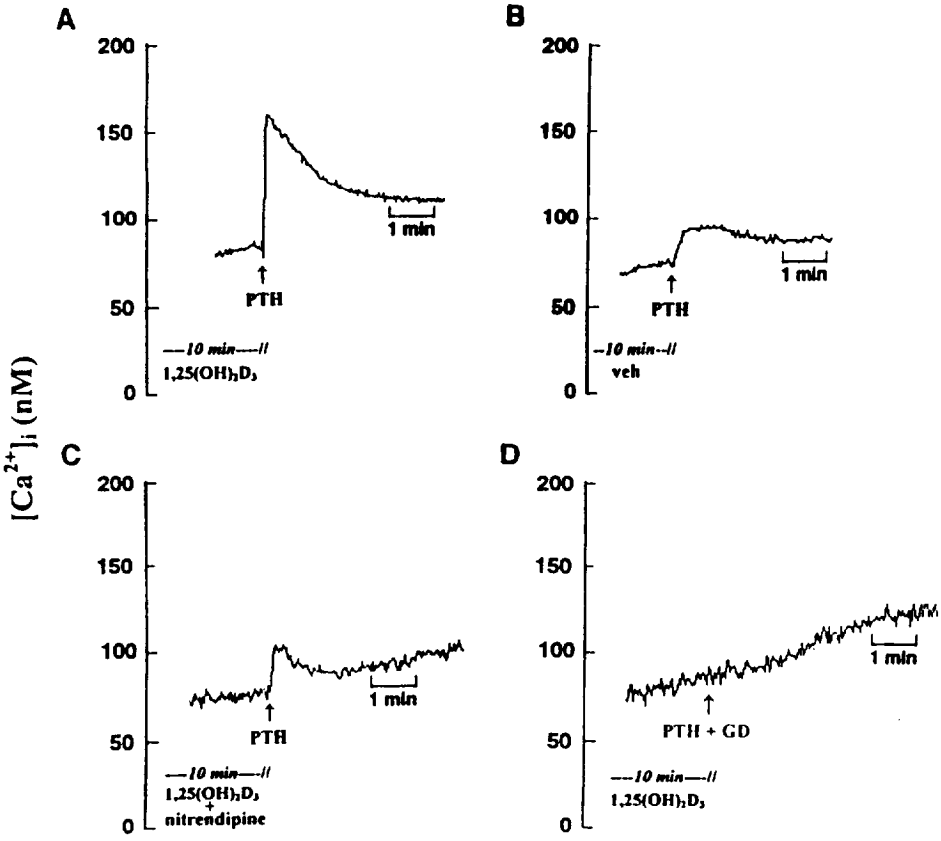

Fig. 2. Enhancement of parathyroid hormone (PTH)-stimulated increase in $[Ca^{+2}]_i$ by $1,25(OH)_2D_3$. MC3T3-E1 cells loaded with fura 2 were pretreated with 10 nM $1,25(OH)_2D_3$ (*A*), ethanol vehicle (*B*), or 10 nM $1,25(OH)_2D_3$ + 5 μM nitrendipine (*C*) for 10 min, at the end of which time PTH (0.5 μM) was added (*arrows*). Comparison of Ca^{2+} transient produced in *A* and *B* demonstrates enhancement effect, which is blocked by inclusion of nitrendipine (*C*). Even after 10 min of pretreatment with $1,25(OH)_2D_3$, PTH-induced Ca^{2+} transient is completely blocked if Gd^{3+} (GD; 10 μM) is added (*D*). This PTH-induced transient is not blocked by nitrendipine (*C*).

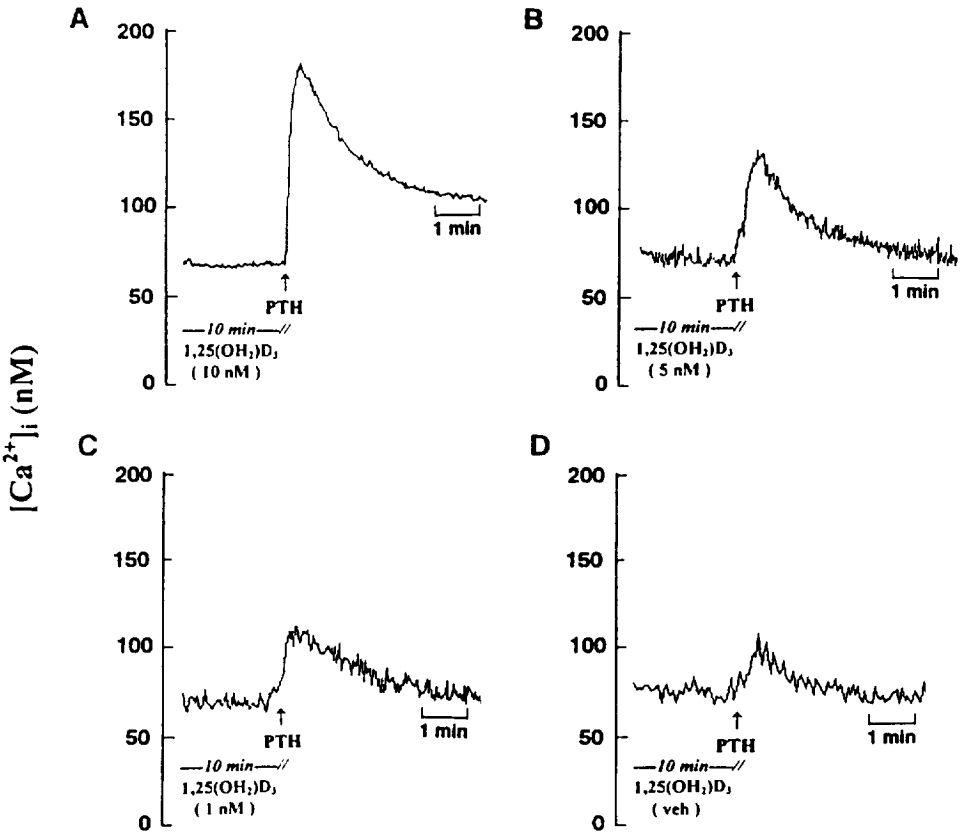

Fig. 3. Ability of 1,25(OH)$_2$D$_3$ to enhance PTH-induced Ca^{2+} currents is dose dependent. MC3T3-E1 cells were treated with various concentrations of 1,25(OH)$_2$D$_3$ for 10 min, then PTH (0.5 μM) was added. Enhancement of PTH-induced Ca^{2+} signal produced by 1,25(OH)$_2$D$_3$ was dose dependent between 1–10 nM. As shown in Fig. 1*B*, this concentration of 1,25(OH)$_2$D$_3$ alone did not produce a Ca^{2+} transient. *A*, 10 nM 1,25(OH)$_2$D$_3$; *B*, 5 nM 1,25(OH)$_2$D$_3$; *C*, 1 nM 1,25(OH)$_2$D$_3$; *D*, vehicle alone.

presumably because they lack appropriate PTH-responsive receptor systems.

Enhancement of the Ca^{2+} transient stimulated by PTH by pretreatment with analog AT but not analog BT. We used two analogs of 1,25(OH)$_2$D$_3$ that selectively activate either genomic nVDR-mediated (analog BT) or nongenomic membrane-initiated Ca^{2+}-signaling pathways (analog AT) in osteoblastic cells (14). We tested the ability of analogs AT and BT to augment the PTH-induced rise in $[Ca^{2+}]_i$ in MC3T3-E1 preosteoblasts, analogous to the previous experiments using 1,25(OH)$_2$D$_3$. Pretreatment of cells with analog AT (10 nM) for 10 min enhanced the Ca^{2+} transient produced by PTH (Fig. 4*A*), which was not seen with the vehicle control (Fig. 2*B*). This enhancement by analog AT was similar in magnitude and duration to that obtained with the parent compound, 1,25(OH)$_2$D$_3$. Conversely, pretreatment with analog BT (10 nM) had no effect on the increase in $[Ca^{2+}]_i$ induced by PTH (Fig. 4*B*). Neither AT nor BT directly produced a rise in $[Ca^{2+}]_i$ in MC3T3-E1 cells in the absence of PTH (data not shown). The inclusion of nitrendipine with analog AT completely negated the enhancement effect (Fig. 4*C*). Inclusion of Gd^{3+} (Fig. 4*D*) also completely abolished the response to PTH after pretreatment with analog AT.

DISCUSSION

1,25(OH)$_2$D$_3$ and PTH are calcitropic hormones that potentiate long-term regulation of bone structure and physiology. This control is exerted, at least in part, by osteoblasts that contain specific receptors for these circulating hormones (6, 26). In the complex processes of bone remodeling and Ca^{2+} homeostasis, the effects of each hormone alone and in combination on the activity of osteoblasts must be considered. In addition to the well-characterized genomic actions attributed to activation of nVDRs, 1,25(OH)$_2$D$_3$ also produces rapid changes in membrane Ca^{2+} permeability that are independent of hormonal regulation of gene expression (8, 16). In this regard, changes in osteoblastic $[Ca^{2+}]_i$ might serve as signals to regulate systemic Ca^{2+} homeostasis by modulating transfer of soluble bone Ca^{2+} to the general extracellular fluid.

Addition of PTH to primary cultures of osteoblasts (17) or to clonal osteoblast-like osteosarcoma cell lines (11, 27, 29) elicits a rapid but transient elevation of $[Ca^{2+}]_i$ that is generated by influx of Ca^{2+} through plasma membrane channels coupled to release of Ca^{2+} from intracellular stores. Furthermore, proliferating cultures of MC3T3-E1 cells express mRNA encoding the PTH receptor, the levels of which increase during cell differentiation (18). In differentiated osteoblasts, 1,25(OH)$_2$D$_3$ also induces rapid increases in $[Ca^{2+}]_i$ by stimulation of transmembrane influx combined with release of Ca^{2+} from intracellular stores (8, 17). Ca^{2+} influx in response to calcitropic agents can be blocked by polyvalent transition metal cations and by several organic Ca^{2+} channel antagonists (8, 17) and thus involves voltage-gated Ca^{2+} channels. Previous studies have also demonstrated that osteoblastic cells express Gd^{3+}-sensitive, stretch-activated channels that can be

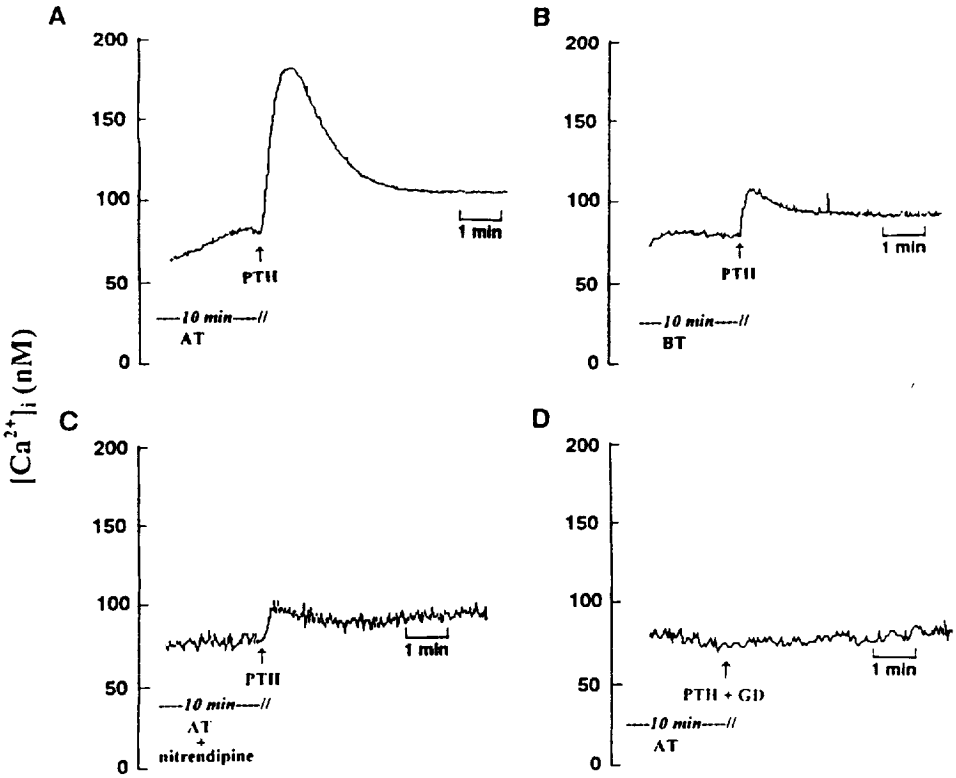

Fig. 4. Enhancement of PTH-stimulated increase in $[Ca^{+2}]_i$ by analog 25-hydroxy-16-ene-23-yne-D_3 (AT) but not analog 1,24-dihydroxy-22-ene-24-cyclopropyl D_3 (BT). MC3T3-E1 cells loaded with fura 2 were pretreated with 10 nM analog AT (*A*) or analog BT (*B*) or 10 nM AT + 5 μM nitrendipine (*C*), after which time PTH (0.5 μM) was added (*arrows*). Effect of AT but not BT was similar to that of 1,25(OH)$_2$D$_3$. AT-enhanced, PTH-induced transient was completely abolished in presence of 10 μM Gd^{3+} (*D*).

stimulated by PTH and provide a large capacitative entry of extracellular Ca^{2+} (12).

In this study, neither 1,25(OH)$_2$D$_3$ treatment (10 nM) nor K^+ depolarization significantly increased $[Ca^{2+}]_i$ in preosteoblastic MC3T3-E1 cells loaded with fura 2, even though intracellular stores were filled. In contrast, an immediate and rapid increase in $[Ca^{2+}]_i$ was produced by addition of 1,25(OH)$_2$D$_3$ to BC_3H_1 premyocytic cells. In previous studies, single-channel measurements performed using a patch clamp revealed that osteosarcoma cells possess about $1-2 \times 10^3$ functional L-type Ca^{2+} channels per cell (4). In comparison, differentiated clonal BC_3H_1 myocytes express $1-2 \times 10^4$ Ca^{2+} channels per cell (2, 25). The very different density of functional Ca^{2+} channels in the plasma membranes of BC_3H_1 myocytes and MC3T3-E1 cells may account for the differences in the response of these two cell types to 1,25(OH)$_2$D$_3$ that we report in Fig. 1. These observations are consistent with the origin of these cell lines in tissues considered to be "excitable" and "nonexcitable," respectively, in which only the former are believed to possess the machinery involved in Ca^{2+}-induced Ca^{2+} release from intracellular stores (9). Supporting this, we previously reported that UMR-106 osteosarcoma cells have no detectable Ca^{2+}-induced Ca^{2+} release (19), assessed by insensitivity to treatment with caffeine. In multiple experiments with fura 2-loaded cells, we were unable to detect transient increases in $[Ca^{2+}]_i$ in proliferating MC3T3-E1 cells treated with 1,25(OH)$_2$D$_3$ alone or subjected to K^+ depolarization. In contrast, Oshima et al. (24) reported transient elevations in $[Ca^{2+}]_i$ in response to 1,25(OH)$_2$D$_3$ but not 24,25(OH)$_2$D$_3$. We believe that this difference reflects a greater state of differentiation in their MC3T3-E1 cultures, which were first grown to confluence, subcultured, then withdrawn from the cell cycle by transfer to low serum medium. We previously found that the steady-state levels of mRNA-encoding L-type Ca^{2+} channels in MC3T3-E1 cells increase substantially during differentiation (20), and it is possible that other systems involved in Ca^{2+}-induced release of Ca^{2+} from stores or regulating Ca^{2+} influx are similarly upregulated.

Earlier studies showed that cells derived from neonatal rat calvaria possess two classes of voltage-gated Ca^{2+} channels of the "low threshold" (T-type) and "high threshold" (L-type) (7), the latter identified by their sensitivity to organic Ca^{2+} channel antagonists, in particular the dihydropyridines. In a previous study (4), our laboratory demonstrated, using single channel recording techniques, that osteoblastic osteosarcoma cells express L-type but not T-type Ca^{2+} channels that respond both to dihydropyridine agonists, such as BAY K 8644, and antagonists, such as nitrendipine. Additionally, we found that, within milliseconds of addition of 1,25(OH)$_2$D$_3$, there consistently occurred a shift in the threshold of activation of inward L-type Ca^{2+} currents to more negative and near-resting potentials, which single-channel analysis revealed was accompanied by a prolonged open time of individual channels (4). On the basis of these findings, we predicted that this shift in the activation threshold would result in an increased responsiveness to other calcitropic hormones, such as PTH, that also activate plasma membrane Ca^{2+} influx through voltage-insensitive channels (12, 13). In this report, we show that in MC3T3-E1 cells, PTH alone stimulates only a modest increase in $[Ca^{2+}]_i$. However,

pretreatment with $1,25(OH)_2D_3$ for 10 min dramatically enhanced the PTH-induced Ca^{2+} transient, clearly indicating that $1,25(OH)_2D_3$ served a priming function to enhance Ca^{2+} responsiveness at the level of the plasma membrane. The need for preincubation with $1,25(OH)_2D_3$ suggests the existence of intracellular pathways involving second messengers, which take minutes to transmit the signal to the PTH-response system. The block of activation by removal of extracellular Ca^{2+} or addition of dihydropyridine channel blockers indicates that enhancement of the PTH response absolutely depends on the presence and influx of extracellular Ca^{2+} through L-type channels.

We previously measured the ability of structural analogs of $1,25(OH)_2D_3$ to stimulate various genomic and plasma membrane-initiated events (14). Although $1,25(OH)_2D_3$ functions as the natural ligand for initiation of both long-term and rapid responses in target cells, we identified subsets of response pathways that were activated by discrete structural analogs. Analog AT activates Ca^{2+} channels in plasma membranes without binding to nVDRs, whereas analog BT binds the nVDR for $1,25(OH)_2D_3$ without triggering a measurable influx of extracellular Ca^{2+}. We examined the effects of analogs AT and BT alone and in combination with PTH on regulation of $[Ca^{2+}]_i$ in MC3T3-E1 cells. Neither AT nor BT alone increased $[Ca^{2+}]_i$ in MC3T3-E1 cells. However, pretreatment with analog AT enhanced the transient rise in $[Ca^{2+}]_i$ stimulated by PTH, consistent with the shift in the threshold of L-type channel activation toward the resting potential ("left shift") reported for this analog in previous studies (30). Unlike analog AT, analog BT did not enhance the PTH-induced elevation in $[Ca^{2+}]_i$. This also is consistent with the inability of this nVDR-selective analog to produce a left shift in the activation threshold at the low nanomolar concentrations used in these studies (30).

Taken together, these data strongly support our hypothesis that $1,25(OH)_2D_3$ and Ca^{2+}-activating analogs serve a priming function by activating plasma membrane voltage-sensitive Ca^{2+} channels. One interpretation of these findings is that voltage-sensitive Ca^{2+} channel activation is required for subsequent full activation of the Gd^{2+}-sensitive channel by PTH, since nitrendipine addition eliminated the enhancement of PTH-sensitive Ca^{2+} influx. A second possibility is that the Gd^{3+}-sensitive channel and the L-type channel must both be activated to generate sufficient depolarization of the plasma membrane to permit development of a large Ca^{2+} transient. In either case, these data indicate the existence of an additional level of interaction of these hormones separate from the previously characterized genomic regulatory loops (5). We believe that this previously unappreciated and novel action of $1,25(OH)_2D_3$ may facilitate the action of other hormones and growth factors acting on osteoblasts and could explain some of the seemingly contradictory physiological effects of $1,25(OH)_2D_3$. It will be of interest to elucidate the mechanism by which Ca^{2+} influx through L-type channels in the plasma membrane is linked to release of Ca^{2+} from intracellular Ca^{2+} stores in osteoblasts and to influx through voltage-insensitive Ca^{2+} channels.

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