1,25(OH)$_2$D$_3$ blunts hormone-elevated cytosolic Ca$^{2+}$ in osteoblast-like cells

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Green, Jacob, Charles R. Kleeman, Sandra Schotland, and Lin Hui Ye. 1,25(OH)$_2$D$_3$ blunts hormone-elevated cytosolic Ca$^{2+}$ in osteoblast-like cells. Am. J. Physiol. 263 (Endocrinol. Metab. 28): E1070–E1076, 1992.—Cytosolic free calcium ([Ca$^{2+}$]) is an important regulator of bone cell physiology. We studied the interaction of vitamin D metabolites on the hormonal-activated Ca$^{2+}$ message system in the osteoblastic cell line UMR-106. The acute rise in [Ca$^{2+}$], induced by different calcitropic hormones [parathyroid hormone, prostaglandin E$_2$ (PGE$_2$)] was dose dependently blunted by 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$; half-maximal inhibitory concn $\sim 5 \times 10^{-11}$ M) and was initially observed after 8 h of preincubation. The 1,25(OH)$_2$D$_3$ metabolite of vitamin D was two orders of magnitude more potent than 24,25(OH)$_2$D$_3$ and 25(OH)D$_3$. To discern between an effect of 1,25(OH)$_2$D$_3$ on hormonally-induced Ca$^{2+}$ entry through the plasma membrane channel vs. release of Ca$^{2+}$ from internal stores, we suspended fura-2-loaded cells in Mn$^{2+}$ rather than Ca$^{2+}$ buffers. In cells preincubated with 1,25(OH)$_2$D$_3$, [Ca$^{2+}$], release (indicated by [Ca$^{2+}$], transient) was significantly blunted, whereas Mn$^{2+}$ influx (indicating Ca$^{2+}$ flux across the plasma membrane) was unaltered, suggesting a selective effect of 1,25(OH)$_2$D$_3$ on hormonally activated release of Ca$^{2+}$ from intracellular stores. 1,25(OH)$_2$D$_3$ also inhibited the PGE$_2$-induced production of inositol 1,4,5-trisphosphate. We conclude that, in osteoblasts, chronic (hours) incubation with 1,25(OH)$_2$D$_3$ leads to attenuated stimulation of the [Ca$^{2+}$], transduction pathway by calcitropic hormones. This effect of 1,25(OH)$_2$D$_3$ may provide a cellular basis for the synergism between the effects of vitamin D and calcitropic hormones at the bone level.

cytosolic calcium ion; 1,25-dihydroxyvitamin D; calcitropic hormones; osteoblasts; phosphoinositols

CONFLICTING DATA exist regarding the interaction of the major calcitropic hormones, 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) and parathyroid hormone (PTH) on their common target organs. Reduced skeletal response to PTH during vitamin D deficiency has been attributed to homologous desensitization resulting from persistent hypocalcemia and chronic elevation of circulating PTH (8). There is also a direct interaction between vitamin D and PTH at the bone cellular level, which can be assessed by using in vitro studies showing modulation of PTH effects on bone cells by vitamin D metabolites. Both PTH and 1,25(OH)$_2$D$_3$ have receptors on the osteoblast, rather than the osteoclast (20, 28). In primary bone cell cultures (16) as well as in osteoblastic cell lines from osteogenic sarcoma (5, 27), 1,25(OH)$_2$D$_3$ has been shown to attenuate the PTH-responsive adenine 3',5'-cyclic monophosphate production. In contrast to the influence of 1,25(OH)$_2$D$_3$ on the adenyl cyclase transduction pathway, there are inconclusive and conflicting data regarding the effect of this hormone on the calcium message system in osteoblasts. In mouse calvaria osteoblasts (17) as well as in osteoblastic cell lines (1, 6, 10, 21), 1,25(OH)$_2$D$_3$ has been shown to induce an instant (within seconds) elevation of cytosolic calcium concentration ([Ca$^{2+}$]) through activation of phospholipase C and generation of inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol. The acute effect of 1,25(OH)$_2$D$_3$ on the Ca$^{2+}$ message system has not been observed in all osteoblastic cell culture systems (9). Moreover, even though elevation of [Ca$^{2+}$], has been established as a major signal transduction pathway for the action of PTH and prostaglandin E$_2$ (PGE$_2$) in osteoblasts (7, 26, 31), there are no data on a possible modulation of these signals by vitamin D metabolites.

Because [Ca$^{2+}$], plays a role in the process of bone resorption (15, 24), we were prompted to define and characterize the influence of vitamin D metabolites on the [Ca$^{2+}$], transduction pathway stimulated by calcitropic hormones in osteoblasts.

For the purpose of this study, we used the osteoblastic cell line UMR-106. These cells display many features of normal osteoblasts (22), including the responsiveness to calcitropic hormones [PTH, 1,25(OH)$_2$D$_3$, and PGE$_2$]; therefore, they can serve as an appropriate model for these studies.

MATERIALS AND METHODS

Culture conditions. The UMR-106 cell line was a generous gift of Dr. T. J. Martin (University of Melbourne, Melbourne, Australia) to Dr. T. J. Hahn (The Veterans Administration Medical Center, West Los Angeles, CA) who in turn generously supplied us with these cells. Cells were used between passages 10–12 and subpassage 3–14. Cells were seeded at a density of 2.5 X 10$^4$ cells/cm$^2$ in tissue culture flasks or multiwell plates and grown at 37°C in a humidified 95% air-5% CO$_2$ atmosphere in Ham’s F$_12$-Dulbecco’s modified Eagle’s media (1:1) supplemented with 14.3 mM NaHCO$_3$, 1.2 mM l-glutamine, 7% fetal bovine serum, 0.1 mg/ml streptomycin, and 100 U/ml penicillin. The cells reached confluence within 5–7 days in culture and were used on day 6–8 of growth.

Determination of [Ca$^{2+}$]. Measurements of free [Ca$^{2+}$], were made by incorporating the Ca-sensitive fluorescent probe fura-2 into UMR-106 cells. Cells grown to confluency were released from tissue culture flasks by trypsin EDTA treatment and then washed and suspended in a balanced salt solution (solution A) containing (in mM) 140 NaCl, 1 MgCl$_2$, 4 KCl, 0.1 Na$_2$HPO$_4$, 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)-tri(hydroxymethyl)amino methane, 1.5 CaCl$_2$, 5 glucose, and 5 sodium pyruvate, pH 7.4 (adjusted with 1 M NaOH). The cells were then incubated with 1 mM fura-2/acetoxymethyl ester (AM) in a shaking water bath at 37°C for 30 min, after which they were washed and resuspended in solution A. Fluorescence was measured in a Perkin-Elmer 650-40 spectrophotometer (Perkin-Elmer, Norwalk, CT) at excitation and emission wavelengths of 340 and 500 nm, respectively, with slits of 3 and 12 nm, respectively. Cells were continuously stirred and kept at 37°C in the spectrophotometer.
Calibration of the fura-2 signal was performed by methods similar to those described for the calibration of another fluorescent \([\text{Ca}^{2+}]\), probe, quin2. Briefly, medium \([\text{Ca}^{2+}]\) was adjusted to 2 mM, and the cells were lysed with digitonin (50 \(\mu\)g/ml) to obtain the maximal fluorescence. Next, 10 mM ethylene glycol-\(\beta\)-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and sufficient NaOH to elevate the pH to 8.5 were added to obtain the minimum fluorescence. The dissociation constant \((K_d)\) for \([\text{Ca}^{2+}]\)-fura-2 was assumed to be 220 nM, and the calculation of \([\text{Ca}^{2+}]\), was similar to that previously described (12). Cells were washed before each experiment, and the above-mentioned calibration for the fura-2 signal was performed after each experiment. To eliminate the effects of autofluorescence due to the cuvette, medium, and cells, fluorescence was measured with an empty cuvette, after addition of medium, and after addition of cells without fura-2. Unloaded cells produced very minimal and almost undetectable fluorescence. Correction for the autofluorescence of the cuvette and medium was made by setting the fluorometer on autozero before each measurement.

To ensure that the various additives to the fluorometer cuvettes did not give nonspecific fluorescence or interact with the dye itself to alter fluorescence independent of changes in \([\text{Ca}^{2+}]\), the tetrapotassium salt of fura-2 in solution A, in the absence of cells or in the presence of unloaded cells, was mixed with concentrations of the various reagents used in these experiments, and the fluorescence was determined. None of the concentrations used in these experiments had any significant effect on dye fluorescence or cell autofluorescence.

**Determination of water-soluble inositol phosphates.** UMR-106 cells in 12 well plates were labeled with 3 \(\mu\)Ci/ml myo\-[\(\text{H}\)]-inositol (Amersham) in Trowell's T8 (inositol free) medium supplemented with 16 mM HEPES, 1.2 mM L-glutamine, 2% fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin for 24 h. On the day of experiment the unincorporated myo-\-[\(\text{H}\)]-inositol was washed away with serum-free Trowell's T8 medium with the above additives and kept in that same medium for 1 h in the presence of 10 mM LiCl. Cells were stimulated with various agonists for the designated times. The reaction was terminated by the aspiration of the medium, followed by two 10% trichloroacetic acid washes. The washes were pooled, and trichloroacetic acid was extracted with water-saturated diethyl ether. The ether extracts were loaded onto glass exchange resin (AG 1-X8 formate, Bio-Rad). Inositol 4-monophosphate (IP1), inositol 1,4-bisphosphate (IP2), and IP3 were sequentially eluted from the columns by increasing concentrations of ammonium formate, according to the method of Berdridge et al. (2). Elution fractions of 2 ml each were diluted into liquid scintillation fluid and counted in a beta counter. Data are expressed as total counts per minute of 6-ml eluates for each inositol phosphate metabolite per 10^6 cells.

**Statistics.** Results are expressed as means \(\pm\) SE. Nonlinear square curve fitting was used to assess dose-response curves to estimate mean half maximally effective (ED50) and maximally effective concentrations of agonist with 67% confidence limits, assuming highly correlated asymmetric variance spaces. One- and two-way analyses of variance to test for differences among treatment means were performed as indicated where appropriate. Each experiment was performed at least four times with separate batches of cells to confirm reproducibility of the results.

**Reagents and hormones.** The vitamin D metabolites, 1,25-(OH)2D3 and 25(OH)D3, were purchased from Biomol Research Lab, except for 24,25(OH)2D3, which was a generous gift from Hoffmann-LaRoche, Nutley, NJ. All metabolites were dissolved in absolute ethanol and added to the cultures so that the final concentration of alcohol in the medium was \(\leq 0.1\%\). PGE2 was obtained from Upjohn (Kalamazoo, MI). Endothelin-1 was obtained from Peptide Institute (Osaka, Japan), and bovine (b) PTH-(1-34) was from Peninsula (San Carlos, CA). Fura-2/AM was obtained from Molecular Probes (Eugene, OR), and phorbol 12-myristate 13-acetate (PMA) was purchased from Calbiochem (San Diego, CA). All other reagents were of the highest purity commercially available.

**RESULTS**

1,25(OH)2D3 influence on agonist-induced \([\text{Ca}^{2+}]\) rise. \([\text{Ca}^{2+}]\), in osteoblasts can be elevated either by opening of a plasma membrane Ca channel (30, 32) or through release of \([\text{Ca}^{2+}]\) from intracellular stores after hormonal activation of phospholipase C and phosphatidylinositol breakdown (7, 26, 31). The following set of experiments describes the influence of 1,25(OH)2D3 on changes in \([\text{Ca}^{2+}]\), caused by different hormonal stimuli.

In Fig. 1, we used bPTH-(1-34) to induce an elevation in \([\text{Ca}^{2+}]\). Under control conditions (Fig. 1A, vehicle-treated cells), acute stimulation with \(10^{-8}\) M PTH in the presence of a 1.5 mM Ca2+ in the medium elicited a rapid rise in \([\text{Ca}^{2+}]\), from a basal value of 102 \(\pm\) 6 nM (n = 10) to a peak level of 188 \(\pm\) 8 nM (n = 10). After the initial transients, \([\text{Ca}^{2+}]\), was reduced although it did not reach the baseline value (141 \(\pm\) 5 nM, n = 8). When cells were preincubated for 24 h with PMA, 1,25(OH)2D3 (Fig. 1A), resting \([\text{Ca}^{2+}]\) was not altered. \([\text{Ca}^{2+}]\), was increased to 106 \(\pm\) 8 nM; P > 0.05 vs. control). The initial \([\text{Ca}^{2+}]\), rise in response to PTH was, however, markedly blunted. Acute stimulation with \(10^{-8}\) M PTH in 1,25(OH)2D3-treated cells increased \([\text{Ca}^{2+}]\), to a peak level of 138 \(\pm\) 6 nM (P < 0.01 vs. control). Figure 1, A and B describe the changes in \([\text{Ca}^{2+}]\), that take place in Ca2+-free media. Under these conditions, any residual Ca2+ in the media was chelated by the addition of EGTA. This resulted in a significant lowering of resting \([\text{Ca}^{2+}]\); due to the fact that, in the

![Fig. 1. UMR 106 cells were preincubated for 24 h with serum free Dulbecco's modified Eagle's medium (DMEM) containing either 10^{-8} M 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) (a and b) or vehicle alone (A and B). On day of experiment, cells were released from tissue culture flasks and loaded with fura-2, as described in MATERIALS AND METHODS. Aliquots of cells were added to 2 ml solution A contained in a cuvette, which was seated in fluorescent spectrophotometer. Parathyroid hormone (PTH)-(1-34) was added (arrows), and fluorescence was recorded. Cytosolic calcium concentration (\([\text{Ca}^{2+}]\)i) was calculated after calibration of dye signal. Experiment was carried out both in presence of 1.5 mM CaCl2 in media (A and B) or in Ca2+-free media containing 0.1 mM EGTA (D and b). This experiment represents 1 out of 10 experiments with similar results.](http://ajpendo.physiology.org/doi/10.1210/ajpendo.107.3.10711)
absence of extracellular Ca\(^{2+}\), [Ca\(^{2+}\)]\(_i\), is pumped out from the cytosol by the plasma membrane Ca\(^{2+}\) adenosinetriphosphatase at a faster rate as compared with when there is Ca\(^{2+}\) in the media. Thus, after 2-3 minutes of incubation in Ca\(^{2+}\)-free media and after the addition of EGTA, [Ca\(^{2+}\)]\(_i\), stabilized at a value of \(-60\) nM. After stabilization of resting [Ca\(^{2+}\)]\(_i\), acute addition of \(10^{-6}\) M PTH to control (vehicle-treated) cells (Fig. 1B) induced [Ca\(^{2+}\)]\(_i\) elevation from a value of \(55 \pm 3\) nM (\(n = 10\)) to \(98 \pm 5\) nM (\(n = 10\)). This rise in [Ca\(^{2+}\)]\(_i\) was due to Ca\(^{2+}\) release from intracellular stores. In cells preincubated for 24 h with 10\(^{-8}\) M 1,25(OH\(_2\))D\(_3\), the PTH-induced [Ca\(^{2+}\)]\(_i\) transient in Ca\(^{2+}\)-free media was completely abrogated (Fig. 1B).

The effect of 1,25(OH\(_2\))D\(_3\) on the hormonal-induced rise in [Ca\(^{2+}\)]\(_i\) was also shown with another hormone that is known to activate the Ca\(^{2+}\) message system in osteoblasts by both opening plasma membrane Ca\(^{2+}\) channels and by releasing Ca\(^{2+}\) from intracellular stores (31). Figure 2 describes changes in [Ca\(^{2+}\)]\(_i\), induced by PGE\(_2\). When 1 \(\mu\)M PGE\(_2\) was acutely added to the cell suspension (control; Fig. 2A) in the presence of 1.5 mM Ca\(^{2+}\) in the medium, [Ca\(^{2+}\)]\(_i\), rose from a resting value of \(115 \pm 6\) nM (\(n = 10\)) to \(162 \pm 3\) nM (\(P < 0.01\) vs. control; Fig. 2a). In Ca\(^{2+}\)-free media, 1 \(\mu\)M PGE\(_2\) induced an elevation in [Ca\(^{2+}\)]\(_i\), from a resting value of \(62 \pm 3\) nM (\(n = 8\)) to \(136 \pm 5\) nM (\(n = 8\); Fig. 2B). In cells preincubated for 24 h with 10\(^{-8}\) M 1,25(OH\(_2\))D\(_3\) (Fig. 2b), PGE\(_2\) stimulated a [Ca\(^{2+}\)]\(_i\) rise from \(62 \pm 6\) nM (\(n = 10\)) to a peak value of \(79 \pm 6\) nM (\(n = 8\); \(P < 0.01\) vs. control).

In contrast to the chronic (24 h) effect of 1,25(OH\(_2\))D\(_3\) on the hormonal-induced [Ca\(^{2+}\)]\(_i\), rise, there was no effect on the hormone, when used alone, on the level of [Ca\(^{2+}\)]\(_i\).

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In contrast to the chronic (24 h) effect of 1,25(OH\(_2\))D\(_3\) on the hormonal-induced [Ca\(^{2+}\)]\(_i\), rise, there was no effect on the hormone, when used alone, on the level of [Ca\(^{2+}\)]\(_i\).

Thus acute addition of 1,25(OH\(_2\))D\(_3\) at a high dose of 10\(^{-8}\) M did not elicit any [Ca\(^{2+}\)]\(_i\) changes (data not shown). Nor was there any change in resting [Ca\(^{2+}\)]\(_i\), after 24 h of preincubation with 1,25(OH\(_2\))D\(_3\).

Thus far, we have demonstrated that 1,25(OH\(_2\))D\(_3\) attenuates [Ca\(^{2+}\)]\(_i\), signals after stimulation with PTH, and PGE\(_2\), both in the presence and the absence of extracellular Ca\(^{2+}\). To distinguish between an effect of 1,25(OH\(_2\))D\(_3\) on Ca\(^{2+}\) entry across the plasma membrane vs. a possible effect on the process of Ca\(^{2+}\) release from intracellular stores, we took advantage of the properties of Mn\(^{2+}\) as a marker of Ca\(^{2+}\) entry. Mn\(^{2+}\) enters the cell through the divalent cation influx pathways and, once inside the cell, the binding of Mn\(^{2+}\) with fura-2 causes quenching of the fluorescence. Cells were suspended in Ca\(^{2+}\)-free medium containing 10 \(\mu\)M Mn\(^{2+}\) (Fig. 3, a and b). Addition of 1 \(\mu\)M PGE\(_2\) stimulated the release of intracellular Ca\(^{2+}\), causing an initial increase in fura-2 fluorescence. This [Ca\(^{2+}\)]\(_i\), rise was significantly blunted in 1,25(OH\(_2\))D\(_3\)-treated cells (10\(^{-8}\) M for 24 h; Fig. 3b) as compared with control cells (Fig. 3a; peak [Ca\(^{2+}\)]\(_i\) = 108 \pm 5 nM vs. 162 \pm 4 nM in 1,25(OH\(_2\))D\(_3\)- and vehicle-treated cells, respectively, \(P < 0.01\). The initial increase in fura-2 fluorescence was followed by fluorescence quenching as Mn\(^{2+}\) entered the cell through the plasma membrane (Fig. 3, a and b). Pretreatment of the cells with 1,25(OH\(_2\))D\(_3\) (Fig. 3b) did not inhibit the fluorescence quenching. It appears, therefore, that the vitamin D metabolite affects Ca\(^{2+}\) release from intracellular stores without altering Ca\(^{2+}\) influx. Figure 3, A and B were used as control experiments to demonstrate lack of fluorescence quenching when Mn\(^{2+}\) is not added to the Ca\(^{2+}\)-free solution. Again the 1,25(OH\(_2\))D\(_3\) effect on the initial Ca\(^{2+}\) transient is clearly shown. The fluorescence quenching by Mn\(^{2+}\), as shown in Fig. 3, a and b, could be blocked by 10 \(\mu\)M La\(^{3+}\) (data not shown). The Mn\(^{2+}\) experiments, as performed with PGE\(_2\), were also carried out with PTH. Similar qualitative results were obtained.
The effect of 1,25(OH)₂D₃ on nonhormonal Ca²⁺ entry mechanisms (i.e., plasma membrane Ca channels) is demonstrated in Fig. 4. We have recently shown that, in addition to a hormonal-mediated Ca²⁺ channel, UMR-106 cells also possess protein kinase C (PKC)-activated and depolarization (=voltage)-activated Ca²⁺ channels (30, 32). When cells were acutely stimulated with 1 μM of the phorbol ester PMA, an activator of PKC, [Ca²⁺]ᵢ rose from a resting value of 123 ± 6 nM to a peak value of 327 ± 8 nM (Fig. 4A). In cells that were incubated with 10⁻⁵ M 1,25(OH)₂D₃ for 24 h, the acute response to the stimulation of PKC was not altered. Figure 4a shows that, under this condition, [Ca²⁺]ᵢ rose from a resting value of 120 ± 6 nM to a peak value of 325 ± 8 nM. To study the effect of 1,25(OH)₂D₃ on the properties of the depolarization-activated Ca²⁺ channel, we acutely exposed the cells to BaCl₂ in Ca²⁺-free media (Fig. 4, B and b). Barium blocks the exit of potassium from the cells, thereby causing membrane depolarization. Cell depolarization activates a voltage-gated channel through which barium (in this case, replacing Ca) can enter the cell. The entry of barium is detected by its binding to fura-2, which elicits a fluorescent signal. Because accurate calculation of the Kᵢ of fura-2 to barium is fraught with great difficulty, it was impossible to calibrate the barium signal. It is, however, clear that, on a qualitative basis, barium entered the cells at a rate and extent that were similar in both control (Fig. 4B) and 1,25(OH)₂D₃-treated cells (Fig. 4b).

The data presented in Figs. 3 and 4, when taken together, indicate that 1,25(OH)₂D₃ modulates [Ca²⁺]ᵢ transients that are stimulated by hormones. The hormone does not affect plasma membrane Ca channels (i.e., hormonal, PKC activated, or voltage activated).

Figure 5 shows the dose-response curve for the suppressive effect of various vitamin D analogues on the acute Ca²⁺ entry in UMR-106 cells. 1,25(OH)₂D₃ had its initial effect at a dose of 10⁻⁹ M, and at a maximum dose of 10⁻⁸ M, it produced a 65% reduction of the [Ca²⁺]ᵢ rise observed at 10⁻⁸ M. 24,25(OH)₂D₃ had its initial effect at 10⁻¹⁰ M, and a maximal response of 23% inhibition of the [Ca²⁺]ᵢ rise was observed at 10⁻⁷ M. 25(OH)D₃ barely showed any effect at all. Figure 5 describes the suppressive effect of vitamin D metabolites on the acute [Ca²⁺]ᵢ rise induced by PGE₂. A similar dose-response curve was obtained when the vitamin D effect was studied in relation to the PTH-induced Ca²⁺ rise (data not shown).

The time course for the modulatory effect of 1,25(OH)₂D₃ on the PGE₂-induced [Ca²⁺] rise is depicted in Fig. 6. The first significant inhibition of the [Ca²⁺]ᵢ rise (28 ± 3%, P < 0.05 vs. control) was observed after 8 h of

![Fig. 4. UMR-106 cells were cultured for 24 h in serum-free DMEM containing either 10⁻⁸ M 1,25(OH)₂D₃ (a and b) or vehicle alone (A and B). On day of experiment, cells were released from tissue culture flasks and loaded with fura-2, as described in MATERIALS AND METHODS. A and a: cells were suspended in 2 ml solution A containing 1.5 mM CaCl₂. Phorbol ester 12-myristate 13-acetate (PMA; 1 μM) was added as indicated, and fluorescence was recorded. B and b: cells were added to 2 ml Ca²⁺-free solution A, and 1 mM BaCl₂ was then added as indicated. This experiment represents 1 out of 5 similar experiments.](image-url)
The inhibitory effect of 1,25(OH)₂D₃ was observed after incubation with 10⁻⁸ M 1,25(OH)₂D₃. The maximum inhibitory effect of 1,25(OH)₂D₃ was observed after 24 h of incubation (60 ± 5%, n = 6).

Figure 7 describes the effect of 1,25(OH)₂D₃ on the dose-response relationship for the PTH-mediated [Ca²⁺]ᵢ rise. As shown in Fig. 7, ED₅₀ for the PTH-stimulated [Ca²⁺]ᵢ rise was not affected by 1,25(OH)₂D₃ (ED₅₀ ~ 4 × 10⁻⁹ M). In contrast, the maximal [Ca²⁺]ᵢ elevation response to PTH was significantly affected by 1,25(OH)₂D₃ (~65% reduction). Similar qualitative results were obtained when a dose-response relationship for PGE₂ was studied. At a maximal dose of 50 μM PGE₂ the [Ca²⁺]ᵢ initial rise was ~60% less in 1,25(OH)₂D₃-treated cells (10⁻⁸ M for 24 h) compared with control. ED₅₀ for the PGE₂-stimulated [Ca²⁺]ᵢ rise was not altered.

Because a release of Ca²⁺ from intracellular stores is closely linked to inositol phosphate turnover, we next measured the effect of 1,25(OH)₂D₃ on phosphatidylinositol metabolism. In Fig. 8, the effect of 1,25(OH)₂D₃ on the production of IP₁, IP₂, and IP₃ mediated by PGE₂ is described. We used PGE₂ as an agonist in this study since we have shown that, in UMR-106 cells, PGE₂ is a potent stimulator of IP₃ generation (31). An increase in IP₃ by PGE₂ was observed after 10 s of stimulation. After 30 s of stimulation, IP₁ was elevated above the control level, whereas IP₃ was undetectable. This finding is probably related to the fact that 30 s is a long enough time during which IP₃ has been already degraded to IP₂ and IP₁. Pretreatment of the cells for 24 h with 10⁻⁸ M 1,25(OH)₂D₃ significantly inhibited inositol phosphate production by PGE₂. IP₃ was 1,485 ± 24 and 482 ± 74 counts·min⁻¹ (cpm)·10⁶ cells⁻¹ in vehicle and 1,25(OH)₂D₃-treated cells, respectively (n = 12, P < 0.01), and IP₁ was 6,405 ± 122 and 2,665 ± 180 cpm/10⁶ cells in vehicle and 1,25(OH)₂D₃-treated cells, respectively (n = 12, P < 0.05). The initial inhibitory effect of 1,25(OH)₂D₃ was observed after 8 h of preincubation, and the overall time course corresponded to the time course of the vitamin D effect on the acute [Ca²⁺]ᵢ rise induced by hormones. Although we did not separate the different IP₃ isomers, these results clearly demonstrate that 1,25(OH)₂D₃ blunts the hydrolysis of phosphatidylinositols triggered by PGE₂, 1,25(OH)₂D₃, when used alone, did not alter the basal level of IP₁ and IP₃ neither after 24 h of preincubation nor after an acute addition to the cells.

**DISCUSSION**

The present studies demonstrate that 1,25(OH)₂D₃ affects [Ca²⁺]ᵢ homeostasis in osteoblasts by attenuating the rise in [Ca²⁺]ᵢ after acute stimulation by calcitropic hormones (PTH, PGE₂). The vitamin D metabolite had a selective effect on the process of [Ca²⁺]ᵢ release from intracellular stores. There was not, however, any influence of 1,25(OH)₂D₃ on plasma membrane Ca²⁺ entry mechanisms. This was true for hormonal-mediated Ca²⁺ influx as well as for PKC and voltage (depolarization)-activated Ca²⁺ channels. 1,25(OH)₂D₃ suppressed the production of inositol phosphates by PGE₂; therefore, it appears that the vitamin exerts its effect on [Ca²⁺]ᵢ, at a level proximal to the generation of IP₃.

1,25(OH)₂D₃ did not have any effect of its own on [Ca²⁺]ᵢ, neither acutely nor after incubation for 24 h. Furthermore, the suppressive effect of 1,25(OH)₂D₃ on the hormonal-induced Ca²⁺ rise was significant only after 8 h of treatment with 10⁻⁸ M 1,25(OH)₂D₃. In this respect, our cell system differs from other osteoblastic cells in which 1,25(OH)₂D₃ has an acute effect on [Ca²⁺]ᵢ. Early
changes (within seconds) in [Ca^{2+}] have recently been reported to occur after 1,25(OH)_{2}D_{3} stimulation of mouse calvaria osteoblasts (17) and in the osteoblastic cell lines MC_{3}T_{3}-E_{1} (21) and ROS 17/2.8 (1, 6, 10). The acute rise in [Ca^{2+}] by 1,25(OH)_{2}D_{3} has been attributed to both the activation of phospholipase C (IP_{3} axis; see Ref. 6) and to activation of dihydropyridine-sensitive Ca^{2+} channels (4). The reason for the lack of an acute effect of 1,25-(OH)_{2}D_{3} in our study is unclear, but it might be related to differences between various cultured osteoblastic cell systems. Moreover, in another study using primary cultures from neonatal rat calvaria, 1,25(OH)_{2}D_{3} did not have any effect on rapid calcium influx (9).

Our study demonstrates that, along with attenuated hormonal-induced [Ca^{2+}] signals by 1,25(OH)_{2}D_{3}, the hormone also suppressed phosphoinosit metabolism. There was a similar time course for both effects of 1,25-(OH)_{2}D_{3}; a significant effect for both was evident after 8 h of treatment with 10^{-6} M 1,25(OH)_{2}D_{3}. This finding, when taken together with the well-known association between Ca^{2+} release from intracellular stores and phosphatidylinositol metabolism (13), suggests a cause and effect relationship between the two effects of 1,25(OH)_{2}D_{3}. Thus, by inhibiting the production of phosphoinosit, 1,25(OH)_{2}D_{3} attenuates the release of Ca^{2+} from intracellular stores induced by various agonists. In this study, we did not determine the mechanism for the suppressive action of 1,25(OH)_{2}D_{3} on the production of phosphoinositols. It is possible that this is related to the effects of 1,25(OH)_{2}D_{3} on plasma membrane phospholipids. Matsumoto et al. (19) examined the effect of 1,25-(OH)_{2}D_{3} on phospholipid metabolism in UMR-106 cells. They found that dose dependently this vitamin D metabolite increased [^{14}C]serine incorporation into phosphatidylserine and decreased [^{3}H]inositol incorporation into phosphatidylinositol. The total content of the phospholipids were similarly affected by 1,25(OH)_{2}D_{3}, suggesting that the vitamin altered the synthesis of these phospholipids. The effect of 1,25(OH)_{2}D_{3} was evident after 8 h. Based on these studies, we hypothesize that, in UMR-106 cells (and by extrapolation in normal osteoblasts), 1,25-(OH)_{2}D_{3} inhibits the synthesis of phosphatidylinositol. This, in turn, leads to reduced generation of IP_{3} upon stimulation with an agonist; therefore, [Ca^{2+}], release from intracellular stores is blunted. We cannot, however, exclude the possibility that 1,25(OH)_{2}D_{3} affected other steps (e.g., hormone-receptor interaction or the G protein function), which could also lead to reduced generation of IP_{3}.

It is of interest to speculate about the biological significance of the blunted hormonal-induced [Ca^{2+}] rise by 1,25(OH)_{2}D_{3}. It is possible that this effect of 1,25(OH)_{2}D_{3} underlies the in situ interaction between vitamin D and other bone-resorbing hormones. It has been recognized for many years that vitamin D-deficient animals show an impaired response to the calcium-mobilizing action of PTH (18, 25). The mechanism responsible for this phenomenon has not been clearly elucidated. It has been suggested that, in vivo, vitamin D deprivation causes homologous desensitization to PTH, which results from chronic elevation of circulating PTH (8). There is most probably, however, a direct effect of vitamin D metabolites on the response to PTH at the bone cell level.

We would like to suggest that the synergistic effect of 1,25(OH)_{2}D_{3} on the bone-resorptive action of PTH (and possibly other bone-resorbing agents as well) derives from its property to blunt the [Ca^{2+}], rise by calcitropic hormones. Elevated calcium in bone has been shown to antagonize the effect of bone-resorbing agents. Thus high Ca^{2+} concentrations applied to bone cultures inhibit bone resorption mediated by PTH and PGE_{2} (14, 15, 24). Assuming that changes in Ca^{2+} in bone culture reflect cytoplasmic changes in bone cells, one may speculate that, by blunting the [Ca^{2+}], rise to PTH (and other bone-resorbing agents), 1,25(OH)_{2}D_{3} potentiates the bone-resorbing activity of PTH. Conversely, vitamin D deficiency could dampen bone responsiveness to bone resorptive hormones in a direct manner. Consistent with this theory is our observation (11) that acute phosphate depletion, which is known to augment bone-resorbing activity by PTH (23), blunts the [Ca^{2+}], rise to PTH, whereas phosphate surfeit, which inhibits PTH-mediated bone resorption (3), augments the [Ca^{2+}], rise to PTH and other calcitropic hormones.

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