**24R,25-(OH)₂ Vitamin D₃ Inhibits 1α,25-(OH)₂ Vitamin D₃ and Testosterone Potentiation of Calcium Channels in Osteosarcoma Cells**

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Calcium influx via L-type calcium channels in osteoblast cells causes a rapid (in seconds) elevation in intracellular calcium initiated by plasma membrane receptors for 1α,25-dihydroxyvitamin D₃ (1α,25-D₃). 24R,25-Dihydroxyvitamin D₃ (24,25-D₃) alone, in concentrations up to 200 nM, does not cause potentiation of calcium currents in osteoblasts, but it does inhibit the current potentiation by 1α,25-D₃. To determine how various steroids interact in their potentiation of calcium channels, the action of vitamin D₃ analogues and testosterone with calcium channels in the rat osteoblast-like cell line ROS 17/2.8 was investigated. Both additions of both 1α,25-D₃ and testosterone at doses below Kᵢₐ (the dose causing 50% left shift in the current-voltage relationship) are additive in their ability to potentiate calcium currents. When 1α,25-D₃ and testosterone are added together at concentrations that would cause a maximal shift in the current-voltage relationship by each agent alone (Vₑₐₓ), the effect of these steroids is not additive. Taken together, these data suggest that potentiation of calcium channels is activated by 1α,25-D₃ or testosterone. The shift in the current-voltage relationship caused by 1α,25-D₃ is reduced by 1β,25-dihydroxyvitamin D₃ (1β,25-D₃), an agent which is thought to act specifically on the plasma membrane receptor for 1α,25-D₃, but the potentiation caused by testosterone is not blocked by 1β,25-D₃. However, 24,25-D₃ inhibits the left shift in the peak current-voltage relationship mediated by either 1α,25-D₃ and testosterone. This result implies that 1) 1β,25-D₃ directly displaces 1α,25-D₃ but not testosterone from its plasma membrane receptor, and 2) the rapid (in seconds) stimulatory effects of 1α,25-D₃ and testosterone on calcium channels are mediated by separate plasma membrane receptors for testosterone and 1α,25-D₃, which are blocked by another receptor for 24,25-D₃. The interaction of these three receptors with L-type calcium channels is pertussis toxin-sensitive.

A newly described function of 1α,25-D₃ and its analogues is the rapid (in seconds) regulation of intracellular calcium concentration, first reported in chicken duodenum (1) and later in bone osteoblast cells (2–12). It has been documented by patch clamp recordings of whole-cell barium currents (2, 3), by ⁴⁵Ca²⁺ uptake (5, 7), and by fluorescence measurements of intracellular calcium, using the calcium indicator acetoxyethyl Quin2 (10), that 1α,25-D₃ (2) and other vitamin D₃ metabolites (3, 5, 7, 8, 12–14) stimulate calcium influx into rat osteosarcoma cells (2, 3, 5, 7, 8, 12, 14) and human and rat osteoblasts (4, 6, 16) in primary culture. Guggino et al. (15) and Caffrey and Farach-Carson (2) have shown the presence of L-type calcium channels in ROS 17/2.8 osteoblast-like osteosarcoma cells. These channels, first described in nerve, brain, cardiac and skeletal muscle, are voltage-dependent, conduct barium and calcium, and are blocked by the dihydropyridine antagonist nifedipine, blocked by the phenylalkylamine antagonist verapamil, and stimulated by the dihydropyridine agonist BayK 8644 (16–19).

Using the patch clamp recording technique, Caffrey and Farach-Carson (2) reported that physiological concentrations of 1α,25-D₃ potentiate L-type calcium channel openings in ROS 17/2.8 cells. The same vitamin D₃ analogues that cause potentiation of calcium channels by the patch clamp technique (3) also cause calcium influx into ROS 17/2.8 cells (5), thus the potentiation of L-type channel current is considered an initial part of the signal transduction process associated with the plasma membrane vitamin D₃ receptors.

The rapid action of 1α,25-D₃ is not associated with steroid binding to a nuclear vitamin D₃ receptor (VDR) binding site because Zhou et al. (13) found several 1α,25-D₃ analogues stimulated ⁴⁵Ca²⁺ transport in duodenum despite poor binding affinity for the intestinal nuclear VDR. Farach-Carson et al. (5) likewise reported that these same analogues stimulated ⁴⁵Ca²⁺ influx into ROS 17/2.8 cells yet bound poorly to the nuclear VDR of ROS 17/2.8. Yukihito et al. (3) reported that the potentiation of calcium channels by these analogues is not compatible with VDR being involved in signal transduction because the structural features like double bonds at carbon 16 and 23 (25-hydroxy-16,23E-diene-D₃) (Fig. 1, A–C) or addition of a hydroxyl group at the 1-carbon position (1-(‘-hydroxyethyl)-25-hydroxy-D₃), which cause activation of rapid calcium signal transduction (5), virtually eliminate binding to VDR (3).

Although it has been reported that 1α,25-D₃ and several analogues potentiate calcium channel openings, some vitamin D₃ analogues with inhibitory effects have also been reported. Norman et al. (8) reported 1β,25-D₃ decreased rapid transcalcitriol stimulated by 1α,25-D₃ in intestine. Yukihito et al. (3) reported that 1β,25-D₃ reduced potentiation of calcium current caused by 1α,25-D₃ and that 24,25-D₃ alone, even in high concentration (200 nM), does not alter calcium currents in ROS 17/2.8 cells, but it reduced the potentiation of calcium channels by the 1α,25-D₃ analogue 1-(‘-hydroxyethyl)-25(OH)₂-D₃. On the other hand, Li et al. (21) reported that 10 μM 24,25-D₃ reduced the amplitude of barium currents in UMR 106 cells, via a protein kinase C-dependent mechanism which takes several minutes to develop.
Androgen receptors have been shown in osteoblast-like cells (22, 23), where, besides the classical functions of sex steroids, rapid actions (in seconds) have also been examined. Lieberherr et al. (10, 11) using fura-2 fluorescence reported that both estrogen and testosterone stimulated calcium influx into osteoblasts which was blocked by calcium channel blockers nitrrendipine and verapamil. She found that rapid actions were also produced by testosterone linked to bovine serum albumin, suggesting that this rapid action was induced by a membrane receptor and not by a nuclear receptor (11). She also found this signal transduction pathway was blocked by pertussis toxin (10, 11).

In order to elucidate the role of testosterone and vitamin D₃ metabolites on potentiation of calcium channels, we asked several questions regarding the relationship between these pathways. 1) Do sex steroids and vitamin D₃ metabolites potentiate calcium channels in the same manner? 2) Do the steroids potentiate the same population of calcium channels? 3) Do steroids act on the same or separate receptors? 4) Are these steroid receptors coupled via G proteins to calcium channels? In this study we tested the interaction of 1α,25-D₃, its analogues, testosterone, and pertussis toxin to evaluate how these steroids potentiate calcium channel function.

MATERIALS AND METHODS

Cell Culture—ROS 17/2.8 cells were grown in 5% CO₂ at 37 °C in Ham's F12 medium (Life Technologies, Inc.) containing 5% heat-inactivated fetal calf serum (Life Technologies, Inc.) and 0.1 mg/ml kanamycin, testosteron, and pertussis toxin to evaluate how these steroid hormones potentiate calcium channel function.

Solutions—The perforated-patch recording technique was used for measuring inward barium currents under voltage clamp conditions. The composition of the pipette solution was 100 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM K₂HPO₄, buffered to pH 7.4 with KOH and the osmolarity adjusted to 290 mosm/kg by using K-Hepes. Amphotericin B, an antibiotic that creates small nonselective pores in the membrane to allow ion flow, was added to this solution at a final concentration of 240 mg/ml, and then the mixture was added to the tip of the pipette. The pipette was then backfilled with pipette saline solution onto the antibiotic saline mixture. The composition of the internal external solution was 140 mM NaCl, 5 mM KCl, 20 mM Hepes buffered to pH 7.4 with NaOH, and the osmolarity adjusted to 290 mosm with Na-Hepes. After whole cell currents were established, a solution which consisted of 115 mM BaCl₂ and 20 mM Hepes buffered to pH 7.4 with tetraethylammonium hydroxide, was added to the initial external solution so the final concentration of barium was 20 mM. Barium was used as a current carrier for two reasons. Barium current through L-type channels is known to be larger than calcium currents, and barium inhibits the activation of potassium channels. External tetraethylammonium was also used as a potassium channel blocker. Hepes, a nonpermeant anion, was used to eliminate inward currents via anion conductance. Using these conditions the inward barium current is stimulated by BayK 8644 (B) and C). BayK 8644 was added at the end of all experiments to verify that the maximal left shift was comparable in all experiments. Peak increase in current occurred 1 min after bath addition of BayK 8644.

Current Measurement—A pipette was placed to the surface of a cell, and then gentle suction was applied until a tight seal of about 10 Gohm was formed. After about 3–10 min the amphotericin B diffused into the cell membrane under the patch pipette causing the capacitance to increase, at which time the experiment was initiated. The cell membrane potential was held at ~70 mV; 10-mV step pulses were applied for 300 ms between ~60 and +50 mV with a 2-s interval between pulses. B-a and B-b, voltage-dependent current traces (control) and in the presence of 1 μM BayK 8644. C, bath addition of 1 μM BayK 8644 (D) caused the peak of the current-voltage relationship to shift negatively along the voltage axis from +23.4 to ~7.6 mV (vertical lines). The amplitude of the current increased in the presence of BayK 8644 (B and C). BayK 8644 was added at the end of all experiments to verify that the maximal left shift was comparable in all experiments. Peak increase in current occurred 1 min after bath addition of BayK 8644.
were recorded just after adding samples already showed about 80% left shift, and the data at 1.5 min showed a maximal steady-state value (Fig. 4). Data were stored and analyzed on a computer (IBM-PC compatible 386, AST) using the P-clamp software version 5.5 (Axon Instruments, Foster City, CA). At the end of each experiment BayK8644 (1 μM) was added to determine the maximal current through the L-type calcium channels and to standardize the data between experiments (Fig. 2). This amount of BayK8644 was previously shown in control cells to cause a maximal increase in barium current and a maximal left shift in barium current. This concentration of BayK8644 usually caused large currents in the range of ~20 mV; thus, as a result of the rather high resistance of the amphotericin patch, the clamp was not totally effective in this voltage range. However, the smaller currents caused by steroids, which were evaluated in this work, were adequately clamped. BayK8644 was used at the end of each experiment to ensure that after three sequential steroid additions the maximal barium currents could be stimulated.

The peak of the current-voltage relationship was estimated by drawing a line joining the three largest current amplitudes. Using an equation that weights the slope of the line on either side of the maximal measured negative current, the peak current was estimated. Thus, the peak current was estimated by the equation 
\[ \text{Current peak} = a - b + c \times 10 \text{ mV} \]
where \( a \) is the maximal current measured by the depolarization protocol; \( b \) is the current measured 10 mV more positive and \( c \) is the current measured 10 mV more negative than maximal current. \( e \) is the difference between the voltage depolarization causing the maximal measured current and 0 mV. All data are represented as mean ± S.E. For statistical analyses, the Student’s t test was used, and \( p < 0.05 \) was recognized as statistically significant.

**RESULTS**

**Effects of Vitamin D3 and Testosterone**—The activation of L-type calcium channels by steroids was investigated using the perforated patch clamp technique. Using 20 mM barium in the external medium to carry inward currents, the maximal potentiation of L-type calcium channels was confirmed at the end of each experiment by the stimulation of currents in response to the dihydropyridine agonist BayK8644 (Fig. 2, B-b and C, Tables I and II). A concentration of 1 μM BayK8644 monitored the maximal peak inward current via L-type calcium channels, as well as determining the maximal left shift in the peak of the current-voltage relationship (Fig. 2). Bath addition of 1α,25-D3 caused the peak of the current-voltage relationship to shift negatively along the voltage axis but did not increase the amplitude of currents (Fig. 3B) as shown previously (3). This left shift in the current-voltage relationship occurred within 30 s of drug application (Fig. 4) (a function of diffusion time of the drug to the cell surface) and remained constant over a period of 30 min, the usual length of the recording time for stable patches. The maximal left shift for 1α,25-D3 was constant for all the experiments recorded in this paper, throughout several different stock tissue culture preparations, and for various serum lots, although the magnitude decreased to 10 mV from values of 20 mV measured earlier by our laboratory (3). 1α,25-D3 caused a left shift in the current-voltage relationship in a dose-dependent manner (Fig. 3B). The averages of the left shifts of the peak currents are shown in Table I. A maximal left shift for 1α,25-D3 (\( V_{\text{max}} \)) was obtained at a concentration of 50 nM (10.9 ± 0.5 mV, \( n = 10 \)) and the \( V_{\text{b}} \), the concentration causing 50% of the maximal left shift of the current-voltage relationship, was 0.37 nM (Fig. 5).

Bath addition of testosterone also caused the peak of the current-voltage relationship to shift negatively in a dose-dependent manner (Fig. 6, Table II). The \( V_{\text{max}} \) of testosterone was slightly higher than 1 nM. The \( V_{1/2} \) was 0.047 nM (Fig. 7).

**The Interaction of 1α,25-D3, Vitamin D3 Analogues, and Testosterone**—We investigated the interaction of these steroids to determine whether their effects on calcium channels were additive. A dose of 0.5 nM 1α,25-D3 (near the \( V_{1/2} \) for 1α,25-D3) and 0.1 nM testosterone (near the \( V_{1/2} \) for testosterone) were additive in their effect on the left shift of the peak of the current-voltage relationship of inward barium current. The total left shift in the current-voltage relationship was 10.1 ± 0.4 mV when 0.1 nM testosterone was added after 0.5 nM 1α,25-D3, which by itself caused an initial left shift of 5.8 ± 0.9 mV (\( n = 5 \)) (Table III, Experiment A). 25(OH)-16,23-E-Diene-D3, a more potent analog of 1α,25-D3, which is specific for activating calcium channels, but has less than 1% binding affinity to VDR (24), has a \( V_{\text{max}} \) of 0.1 nM (data not shown). A concentration of testosterone near the \( V_{1/2} \) (0.1 nM) added after 0.05 nM 25(OH)-16,23E-diene-D3 (also near the \( V_{1/2} \) (Fig. 8) is additive in its ability to shift the peak of the current-voltage relationship of the inward barium current (Table III, Experiment B). However, when 10 nM 1α,25-D3 (a \( V_{\text{max}} \) dose) and 1 nM testosterone (a \( V_{\text{max}} \) dose) were added together, the left shift in the peak of the current-voltage relationship was no larger than that present when each agent was added alone (Table III, Experiment C). For example, 10 nM 1α,25-D3 followed by 1 nM testosterone caused a left shift of 9.8 ± 0.5 mV (\( n = 3 \)) compared with a left shift of 9.1 ± 0.9 mV (\( n = 3 \)) when 1α,25-D3 was added alone (Table III, Experiment C). 100 nM 1β,25-D3 has been shown previously to reduce the potentiation of calcium channels by 1α,25-D3 (3). 1β,25-D3 by itself does not cause a left shift of the peak of inward barium currents at a concentration of up to 100 nM (Table IV, Experiments A and B, line 1) (3), but the left shift in the current-voltage relationship was reduced by 1β,25-D3, to 32% of control for 1α,25-D3 and 47% of control for 25(OH)-16,23E-diene-D3 (Table IV, Experiments A and B). On the other hand, the left shift in the current-voltage relationship caused by testosterone was maintained constant even when 100 nM 1β,25-D3 was

**TABLE I**

| 1α,25-D3 | 0.5 nM | 5 nM | 50 nM | BayK 8644, 1 μM | No. of experiments |
|----------|--------|------|-------|----------------|-------------------|
| Peak shift (mV) | 5.6 ± 0.9 | 10.0 ± 0.2 | 10.9 ± 0.5 | 33.4 ± 2.1 | 10 |

**TABLE II**

| Testosterone | 0.1 nM | 1 nM | 10 nM | BayK 8644, 1 μM | No. of experiments |
|--------------|--------|------|-------|----------------|-------------------|
| Peak shift (mV) | 6.6 ± 0.9 | 9.7 ± 1.0 | 11.7 ± 1.0 | 33.4 ± 1.5 | 12 |
FIG. 3. Whole cell recordings of barium currents in the absence (control) and the presence of 1α,25-dihydroxyvitamin D₃. A, the pulse protocol was the same as in Fig. 2A, but only the step pulses between 0 and +40 mV are shown for clarity. B-a are representative control current traces (top) and the resulting current-voltage relationship (below). B-b–d are the current traces and the current-voltage relationships of the same cell as B-a given three increasing, sequentially added concentrations of 1α,25-D₃. Bath addition of 1α,25-D₃ caused, in a dose-dependent manner, the peak current of the current-voltage relationship to shift from +25 mV in control to more negative values along the voltage axis. The vertical line shows the peak of the current-voltage relationship for each concentration of 1α,25-D₃ (+25.0 mV for control; +19.1 mV for 0.5 nM 1α,25-D₃, a left shift of −5.9 mV; +15.0 mV for 5 nM 1α,25-D₃, a left shift of −10.0 mV; and +14.6 mV for 50 nM 1α,25-D₃, a left shift of −10.4 mV). The magnitude of the current did not increase with addition of 1α,25-D₃. The symbols for each voltage are as follows: ● 0 mV; ◆ 10 mV; ▲ 20 mV; ▣ 30 mV; ▼ 40 mV (B-b–d). Current traces shown were measured 1.5 min after addition of steroids, when the maximal left shift in current occurs (Fig. 4).

24,25-(OH)₂ Vitamin D₃ Regulates Calcium Channels

24,25-D₃, in concentrations up to 200 nm, did not cause a left shift in the current-voltage relationship when added alone (Table V, Experiments A and B, line 1) (3). We tested the effect of 24,25-D₃ on calcium channel potentiation produced by other steroids. When 100 nm 24,25-D₃ was added to 5 nm 1α,25-D₃, the resulting left shift of the peak current-voltage relationship was only 4.9 ± 1.1 mV (n = 4) (Table V, Experiment A, line 2) as compared with 9.7 ± 0.9 mV (n = 5) when 1α,25-D₃ was applied alone (Table V, Experiment A, line 5). Likewise, the left shift produced by 0.1 nm 25(OH)-16,23E-diene-D₃ was reduced to 48% of control when 24,25-D₃ was added to the bath before 25(OH)-16,23E-diene-D₃ (Table V, Experiment A, line 5).

Therefore, the presence of 24,25-D₃ blunted the left shift of the current-voltage relationship caused by either 1α,25-D₃ or 25(OH)-16,23E-diene-D₃. Similarly, 24,25-D₃ reduced the left shift in the current-voltage relationship caused by testosterone (Table V, Experiment D). When 100 nm 24,25-D₃ was added before 0.1, 1.0, or 10 nm testosterone, the left shift at each concentration was significantly decreased (p < 0.05). The final left shift, when 100 nm 24,25-D₃ was added in combination with testosterone, was 2.6 ± 1.3 mV for 0.1 nm testosterone, 4.2 ± 1.1 mV (n = 4) for 1 nm testosterone, and 7.6 ± 0.7 mV (n = 4) for 10 nm testosterone, respectively (Table V, Experiment D), as compared with 6.6 ± 1.5 mV (n = 4) for 0.1 nm, 9.7 ± 0.1 mV (n = 4) for 1 nm, and 10.1 ± 0.5 mV (n = 4) for 10 nm when testosterone was added alone (Table V, Experiment C). Thus, regardless of whether testosterone was added below the V₁/₂ or severalfold above the V₁/₂, 24,25-D₃ effectively decreased the left shift in barium current.

Effects of Pertussis Toxin (PTX) on the Potentiation Caused
by 1α,25-D3, 24,25-D3, Testosterone—We investigated the effects of PTX to determine whether the potentiation of calcium channels caused by steroids was mediated via G-proteins. Cells were preincubated for 15–30 min with 500 ng/ml PTX, and then 5 nM 1α,25-D3 (Vmax dose) or 1 nM testosterone (Vmax dose) was added to the cell as described previously. The cells preincubated with PTX showed a decreased left shift in the current-voltage relationship caused by 1α,25-D3 or testosterone. For 1α,25-D3, PTX reduced the left shift to under 30% of that caused by 1α,25-D3 alone in 7/14 cells. The average left shift of 5.0 ± 0.9 mV (n = 14) caused by 1α,25-D3 in cells preincubated with PTX was significantly different (p < 0.01) when compared with the left shifts seen in the cells without preincubation (Table VI, Experiment A). For testosterone, PTX reduced the left shift to under 50% of that caused by testosterone alone (4/15 cells). The average left shift of 7.0 ± 0.8 mV (n = 15) caused by testosterone in cells preincubated in PTX was significantly different (p < 0.05) compared with control group (Table VI, Experiment B). Under normal circumstances 24,25-D3 does not cause a left shift in the current-voltage relationship (1.4 ± 0.5 mV) (n = 6), but cells preincubated in PTX showed a left shift of 6.6 ± 1.4 mV in 8/9 cells (Table VI, Experiment C). The left shift was significantly different (p < 0.01) compared with the control group. All PTX-treated cells had normal responses to BayK 8644.

**DISCUSSION**

Intracellular calcium concentration in most cells is regulated by the release of calcium from intracellular calcium storage compartments (25, 26) and calcium influx through calcium channels (2, 15, 27, 28). The rapid increase of intracellular calcium concentration caused by vitamin D3 analogues and other steroid hormones is an area of increasing investigation (2–13, 29, 30). 1α,25-D3 and its analogues rapidly activate inward calcium movement in osteoblasts or osteoblast-like cells (2–7, 12). Using acetoxymethyl Quin2 fluorescent calcium indicator, Lieberherr (6) concluded, based on inhibition by dihydropyridine drugs like nitrendipine, that 70% of the increase in intracellular calcium concentration caused by 1α,25-D3 was from calcium influx via L-type calcium channels, suggesting this is a major pathway for calcium influx. Furthermore, she found that testosterone and estrogen also caused increases in intracellular calcium (10, 11). We have verified by the patch clamp technique that 1α,25-D3 and testosterone both act by the same mechanism (a left shift in the current-voltage relationship) to potentiate the opening of L-type calcium channels.
24,25-(OH)$_2$ Vitamin D$_3$ Regulates Calcium Channels

To determine whether calcium movement is initiated by the well characterized nuclear steroid receptor, VDR, or a totally separate plasma membrane receptor, 1a,25-D$_3$ and its analogues have been tested on transepithelial movements of $^{45}$Ca$^{2+}$ in intestine (transcaltachia) (1, 5, 7, 20) or $^{45}$Ca$^{2+}$ influx (2, 5, 7, 12) or whole cell currents (2–4) in osteosarcoma cells. The OH group at the 1a position of 1a,25-D$_3$ is well known to confer potency on VDR (29–31) (Fig. 1A). However, other parts of the vitamin D$_3$ structure like a double bond in the C/D side chain (Fig. 1, A–C) are permissive for the rapid movements of calcium associated with L-type calcium channels. Zhou et al. (13), Farach-Carson et al. (5), and Yukihiro et al. (3) reported stimulatory effects of analogues with double bonds at the 23-carbon position. These data indicate that synthetic analogues that stimulate calcium influx have different structural features that are not compatible with an affinity at the nuclear VDR binding site. Current thinking suggests that regulation of calcium channel openings may be initiated by a different ligand binding receptor from that which initiates the genomic action. Thus, in order to eliminate the possibility of a VDR-type binding site, we verified all experiments involving 1a,25-D$_3$ with the analogue 25(OH)(16,23)E-diene-D$_3$ which acts only to stimulate calcium channels and has minimal binding to VDR (24).

Unlike BayK 8644, these steroids do not increase the amplitude of currents but rather shift the current-voltage relationship (Figs. 3B and 6 and Tables I and II), suggesting a mechanism of action on calcium channels which involves alteration in the voltage dependence of the channel. In addition, 1a,25-D$_3$, 25(OH)(16,23)E-diene-D$_3$, and testosterone all had the same $V_{max}$ for the left shift, suggesting they alter the voltage dependence by the same amount. The fact that these steroids all have about 1⁄3 the efficacy of BayK 8644 implies that they are less potent than dihydropyridine agonists in potentiation of total calcium channel current or that they act only on a portion of the population of all calcium channels. We investigated the effect of combinations of 1a,25-D$_3$, its analogues, and testosterone on calcium channel currents in rat osteoblast-like cells in order to better understand the interaction between the steroid receptors and the calcium channels they modulate. 1a,25-D$_3$, 25(OH)(16,23)E-diene-D$_3$, and testosterone, when supplied at doses near the $V_{1/2}$, add together to shift the peak of the current-voltage relationship (Table III, Experiments A and B), but when given at their $V_{max}$ concentration their effects are not additive (Table III, Experiment C), implying that these agents converge on the same population of calcium channels.

Norman et al. (8, 9) reported the inhibitory effect of 1β,25-D$_3$ on the transcaltachia stimulated by 1a,25-D$_3$. Yukihiro et al. (3) demonstrated the inhibitory effect of 1β,25-D$_3$ on the peak shift of the current-voltage relationship of currents potentiated by 1a,25-D$_3$. Therefore, we evaluated effects of 1β,25-D$_3$ on the potentiation caused by 1a,25-D$_3$, 25(OH)(16,23)E-diene-D$_3$, and testosterone. 1β,25-D$_3$ decreased the left shift in the calcium channel current-voltage relationship potentiated by 1a,25-D$_3$ or 25(OH)(16,23)E-diene-D$_3$ but did not alter the potentiation caused by testosterone (Table IV). Because 1β,25-D$_3$ is very similar to 1a,25-D$_3$ in structure, it may block directly at the plasma membrane 1a,25-D$_3$ receptor but does not compete at the plasma membrane testosterone receptor which probably has a very different binding site.

Interestingly, 24,25-D$_3$, by itself, stimulates transcaltachia in the duodenum (30). In ROS 17/2.8 cells, 24,25-D$_3$ inhibits $^{45}$Ca$^{2+}$ influx and reduces whole-cell barium currents at very positive membrane potentials when concentrations greater than 1.0 nm are added to the bath (5). In patch clamp experiments measuring barium currents of ROS 17/2.8 cells, 24,25-D$_3$ caused no left shift of the peak of the current-voltage relationship when added in concentrations between 0.5 and 200 nm, although it does decrease calcium currents at very positive membrane potentials (3). In UMR 106 cells, 10 nm 24,25-D$_3$ increased the amplitude of barium current, whereas a high concentration (10 μm) of 24,25-D$_3$ reduced the amplitude of the
current (21). The left shift in current-voltage relationship caused by 24,25-D₃ occurs within 10–15 min, probably to involve phosphorylation of calcium channel by protein kinase A and C pathway (21). Thus, 24,25-D₃, by itself, can decrease the left shift caused by 1α,25-D₃ (Experiment D) when compared with cells which received testosterone alone even when testosterone was added at doses in 10-fold excess of saturation concentration needed to cause a left shift in the current-voltage relationship (Experiment C). 1 μM Bay K 8644 added at the end of these experiments produced consistent effects (35.3 ± 1.1, n = 11). When the results were compared using a Student’s test, c and d were significantly different at p < 0.01 and p < 0.05, respectively, but e, f, and g were not statistically different.

### Table IV

| Experiment | No. of experiments (n = x) | Peak shift (mV) | Average peak shift compared to control (mV) |
|------------|----------------------------|----------------|------------------------------------------|
| A         | 100 nM (alone)             | 0.4 ± 0.9ₚ     |                                           |
| 100 nM     | 5 nM with 1α,25-D₃ 100 nM  | 3.0 ± 0.7ₚ     |                                           |
| Bay K 8644 | 1 µM                       | 32.5 ± 1.0     |                                           |
| B         | 1α,25-D₃ 5 nM (alone)      | 9.5 ± 0.4ₚ     |                                           |
| C         | 1α,25-D₃ 100 nM (alone)    | 1.1 ± 0.3ₚ     |                                           |
| 25(OH)-16,23E-diene-D₃ 0.1 nM with 1α,25-D₃ 100 nM | 4.4 ± 1.4ₚ |                                           |
| Bay K 8644 | 1 µM                       | 31.3 ± 1.3     |                                           |
| D         | 25(OH)-16,23E-diene-D₃ 0.1 nM (alone) | 9.3 ± 0.4ₚ |                                           |

### Table V

| Experiment | No. of experiments (n = x) | Peak shift (mV) | Average peak shift compared to control (mV) |
|------------|----------------------------|----------------|------------------------------------------|
| A         | 100 nM (alone)             | 0.6 ± 0.2ₚ     |                                           |
| 1α,25-D₃ 5 nM with 24,25-D₃ 100 nM | 4.9 ± 1.1ₚ |                                           |
| Bay K 8644 | 1 µM                       | 27.6 ± 3.2     |                                           |
| C         | 1α,25-D₃ 5 nM (alone)      | 9.7 ± 0.9ₚ     |                                           |
| 24,25-D₃ 100 nM (alone) | 1.1 ± 0.4ₚ |                                           |
| 25(OH)-16,23E-diene-D₃ 0.1 nM with 24,25-D₃ 100 nM | 4.5 ± 1.4ₚ |                                           |
| Bay K 8644 | 1 µM                       | 34.5 ± 1.3     |                                           |
| D         | 25(OH)-16,23E-diene-D₃ 0.1 nM (alone) | 9.3 ± 0.4ₚ |                                           |

**Inhibitory effect of 24,25-dihydroxyvitamin D₃ on agonist-stimulated calcium currents**

Experiments A and B, in these experiments 100 nM 24,25-D₃ was added to the bath first and then 5 nM 1α,25-D₃ (at Vₘₐₓ dose) or 0.1 nM 25(OH)-16,23E-diene-D₃ (at Vₘₐₓ dose) was added to the bath afterward. 100 nM 1α,25-D₃ did not cause a left shift in the current-voltage relationship when added by itself (line 1). 1α,25-D₃, decreased the left shift caused by 1α,25-D₃, or 25(OH)-16,23E-diene-D₃ (line 2) compared with the current-voltage relationship measured in cells to which the agent was added alone (line 5). Experiment C, in these experiments testosterone was added alone. The peak left shift was increased by testosterone in a dose-dependent manner. Experiment D, 100 nM 1α,25-D₃, was added to the bath first, then testosterone was added to the bath afterward. The shift in current-voltage relationship was compared for each concentration of testosterone. The peak-shift induced by testosterone was not decreased by 1α,25-D₃ (Experiment D) when compared with cells which received testosterone alone even when testosterone was added at doses in 10-fold excess of saturation concentration needed to cause a left shift in the current-voltage relationship (Experiment C). 1 μM Bay K 8644 added at the end of these experiments produced consistent effects (35.3 ± 1.1, n = 11). When the results were compared using a Student’s test, c and d were significantly different at p < 0.01 and p < 0.05, respectively, but e, f, and g were not statistically different.
TABLE VI

| Vitamin D3 Metabolites | Testosterone | Calcium Channel Potentiation (n = 4) |
|------------------------|-------------|-------------------------------------|
| 1,25-(OH)2 D3         | 1,25-(OH)2 D3 | 1.2 mV |
| 24,25-(OH)2 D3        | 1,25-(OH)2 D3 | 1.1 mV |
| 24,25-(OH)2 D3        | Testosterone  | 1.0 mV |
| 24,25-(OH)2 D3        | BayK 8644    | 1.0 mV |
| 24,25-(OH)2 D3        | PTX         | 1.0 mV |

In the control group (Experiment A), 5 nm 1α,25-D3 (at Vmax dose) was added to the bath without preincubation of PTX. 5 nm 1α,25-D3 caused 9.4 ± 0.2 mV left shift in the current-voltage relationship (line 1). In the PTX-treated group, cells from the same pass and split were preincubated with 500 ng/ml PTX for 15–30 min, and then 5 nm 1α,25-D3 (at Vmax dose) was added to the bath. Some cells preincubated with PTX showed a decrease in the left shift caused by the addition of 1α,25-D3 (line 2) when compared with control (line 1). PTX reduced the magnitude of the left shift to under 50% of control in 7/14 cases. The left shift caused by 1 μM BayK 8644 at the end of these experiments was 39.3 ± 0.9 mV when 5 nm 1α,25-D3 + PTX where added together. When Student's test was used to compare 1α,25-D3 alone and 1α,25-D3 with PTX, the differences were significant. Experiment B, in the control group, 1 nm testosterone (at Vmax dose) was added to the bath without preincubation of PTX. 1 nm testosterone caused 9.1 mV left shift in the current-voltage relationship (line 1). In the PTX-treated group, cells were preincubated with 500 ng/ml PTX for 15–30 min, and then 1 nm testosterone (at Vmax dose) was added to the bath afterward. Some cells preincubated with PTX showed a decrease in the left shift caused by the addition of testosterone when compared with control which was measured in other cells from the same pass and split. PTX reduced the magnitude of the left shift to under 50% in 4/15 cases. The left shift caused by 1 μM BayK 8644 at the end of these experiments was 33.8 ± 1.1 mV when 5 nm 1α,25-D3 + PTX where added together. When Student's test was used to compare the left shift caused by 1α,25-D3 alone compared to 1α,25-D3 with PTX, the differences were significant.

Acknowledgment—We thank Dr. Shigeumi Yukihiro for helpful discussions regarding data analysis.

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