Vitamin D₃ Metabolites Modulate Dihydropyridine-sensitive Calcium Currents in Clonal Rat Osteosarcoma Cells*

(Received for publication, May 24, 1989)

John M. Caffrey‡§ and Mary C. Farach-Carson¶¶

From the ‡Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, Texas 77030 and the ¶¶Department of Biological Chemistry, University of Texas Dental Branch, Houston, Texas 77225

A slowly inactivating inward calcium current was identified in the rat osteosarcoma cell line ROS 17/2.8 using a combination of ion flux and electrophysiological techniques. Voltage dependence, dihydropyridine sensitivity, divalent cation selectivity, and single channel properties identified this current as a high threshold, "L-type" calcium current. Ion flux experiments using ⁴⁰Ca⁺⁺ confirmed that calcium uptake through these channels represents a major pathway for calcium entry into osteosarcoma cells. In resting cells, i.e. at negative membrane potentials, stimulation of both calcium current and rapid ⁴⁰Ca⁺⁺ influx could be elicited by concentrations of 1,25-(OH)₂-vitamin D₃ between 0.1 and 3 nM. At these concentrations, 1,25-(OH)₂-vitamin D₃ shifted the threshold for activation of inward calcium current to more negative potentials. At higher concentrations (5–10 nM), inhibitory effects became predominant. These opposing effects are functionally similar to those of the dihydropyridine BAY K 8644. Other vitamin D₃ metabolites (25-(OH)-D₃ and 24,25-(OH)₂-D₃) exhibited less potent stimulatory effects and greater inhibition of calcium current than 1,25-(OH)₂-D₃. These results suggest that (i) vitamin D₃ acts as a potent modulator of calcium channel function in osteosarcoma cells, and (ii) intracellular Ca²⁺-dependent signaling processes may be affected acutely by physiological concentrations of vitamin D₃ metabolites.

Recent studies have shown that bone remodeling is influenced by a variety of calcitropic hormones and cytokines that act directly on the osteoblast (for a review, see Ref. 1 and references therein). These include both steroid (vitamin D₃, estrogen) and peptide (parathyroid hormone (PTH), calcitonin) hormones as well as autocrine/paracrine agents such as transforming growth factor β and granulocyte/macrophage colony-stimulating factor (2). Previous studies have demonstrated that many of these agents function as activators or inhibitors of bone-specific genes such as collagen type I (3, 4), osteopontin (5, 6), or osteocalcin (7, 8). These long term regulatory events are potentiated by the activation of DNA-binding proteins/nuclear receptors which initiate transcription of target genes (1). Physiological concentrations of calcitropic agents may also induce short term changes in membrane permeability and activate intracellular signaling pathways. In addition to messengers generated by enzyme activation such as diacylglycerol, cAMP or the phosphoinositides (9–11), elevation of intracellular Ca²⁺ levels in response to external stimuli can exert both direct and indirect regulatory effects. In this regard, Ca²⁺ might serve as a proximal "short loop" feedback signal regulating osteoblast control of deposition or resorption of extracellular calcified matrix.

Recently, Chesnoy-Marchaise and Fritsch (12) have demonstrated that cells derived from neonatal rat calvaria possess voltage-gated, tetrodotoxin-sensitive Na⁺ channels and two classes of voltage-gated Ca²⁺ channels. These Ca²⁺ channels were of the "low threshold" (T-type) and "high threshold" (L-type) (13, 14), the latter identifiable by their sensitivity to organic Ca²⁺ channel antagonists, in particular the dihydropyridines (DHPs). Furthermore, Guggino et al. (15) reported the presence of L-type Ca²⁺ channels in osteoblast-like osteosarcoma cells using whole cell recording techniques.

Addition of PTH to primary cultures of osteoblasts (16) or to clonal osteoblast-like osteosarcoma cell lines (17–19) elicits a rapid, but transient, elevation of intracellular Ca²⁺ that appears to be generated by influx of Ca²⁺ through the plasma membrane. Vitamin D₃ metabolites and prostaglandins (16, 20) can also induce rapid increases in cytosolic Ca²⁺ concentrations via transmembrane influx, although in certain cases this may also involve release of Ca²⁺ from intracellular stores. Extracellular Ca²⁺ influx in response to calcitropic agents can be blocked by polyvalent transition metal cations and by several of the organic Ca²⁺ channel antagonists (16, 19, 21) and thus may be mediated by voltage-gated Ca²⁺ channels. Using the calcium-sensing dye Quin 2, Lieberherr (16) showed that 1,25-(OH)₂-D₃, 24,25-(OH)₂-D₃, and 25-(OH)-vitamin D₃ metabolites rapidly induced significant increases in intracellular Ca²⁺ in primary cultures of osteoblasts. 1,25-(OH)₂-D₃ was the most potent, eliciting a maximum response at concentrations of 10–100 pM. Responses to 1,25-(OH)₂-D₃ could be entirely eliminated by reduction of extracellular Ca²⁺ and by Ca²⁺ channel blockers. In contrast, elevation of intracellular Ca²⁺ in response to 24,25-(OH)₂-D₃ and 25-(OH)-vitamin D₃ could only be partially blocked by these treatments, suggesting a significant contribution of release from intracellular stores. The potency of action of 1,25-(OH)₂-vitamin D₃ on a DHP-sensitive Ca²⁺ influx pathway in bone cells raises the possibility of direct action(s) of vitamin D₃ metabolites on voltage-sensitive Ca²⁺ channels. The osteoblast-like osteosarcoma cell
line ROS 17/2.8 is responsive to both PTH and vitamin D₃ (22, 23) and is thus a useful model system for investigating these regulatory effects. In this report, we show that the only detectable voltage-gated inward current in ROS 17/2.8 cells is generated by a DHP-sensitive, "L-type" calcium channel that is virtually indistinguishable from that characterized in other excitable cells. The modulation of this channel by vitamin D₃ metabolites is described.

MATERIALS AND METHODS

Cell Culture—ROS 17/2.8 cells (kindly provided by Dr. Gideon Rodan, Merck Sharp and Dohme, West Point, PA) were cultured in Ham's F-12 medium containing 5% fetal calf serum and 5% Serum Plus (Hazelton, Lenexa, KS). Medium was supplemented with 1.1 mM CaCl₂ as described (23). For "Ca²⁺" uptake experiments, cells were seeded at a density of 50,000 cells/ml into 3.5-cm dishes and grown to approximately 50% confluency. For electrophysiological experiments, cells were plated at low density (25,000 cells/plate) onto 1-

RESULTS

ROS 17/2.8 Cells Possess L-type Ca²⁺ Channels—The electrical properties of ROS 17/2.8 cells were examined both electrophysiological and ion flux assays. Using internal and external solutions designed to isolate Ca²⁺ currents, it was found that these cells express a slowly inactivating inward current that demonstrates biophysical properties identifying it as an L-type Ca²⁺ current (defined in Ref. 13). In external solutions containing normal concentrations of Na⁺ and Ca²⁺, neither an inward Na⁺ current nor a transient low threshold "T-type" Ca²⁺ current was present (data not shown). As seen in Fig. 1, A, C, and D, whole cell recordings of patch-clamped ROS 17/2.8 cells revealed an inward current in 20 mM Ba²⁺ that was activated by depolarization of membrane potential to voltages between −20 and +20 mV. Maximum inward current was elicited at approximately +15 mV. No outward current was detectable at very positive potentials (> +60 mV), indicating that dialysis of intracellular K⁺ and buffering of internal Ca²⁺ by EGTA was sufficient to eliminate contribution of K⁺ currents. The voltage dependence of activation was measured using tail currents following cessation of the test depolarization at a repolarization potential of −50 mV. Normalized amplitude of tail currents as a function of test potential could be fit by a first power Boltzmann distribution: Mₐ = [1 + exp(Vₜ−Vₚ/κ)]⁻¹ in which Vₜ = 0 ± 2.5 mV and κ = 12 ± 2 mV (n = 6) (Fig. 1D). Steady state activation or current availability was measured using changes in holding potential initiated 30 s to 1 min preceding a depolarizing test pulse to +10 mV (Fig. 1B). Normalized peak current amplitude during the test pulse could be fit by a Boltzmann distribution: Hₚ = [1 + exp(Vₜ−Vₚ/κ)]⁻¹ in which Vₚ = −15 ± 4.5 mV and κ = 6 ± 2 mV (n = 6). Since steady state current is empirically defined as the product of the Mₐ and Hₚ parameters, these data define a membrane potential range within which steady state ("window") current is possible. Steady state inward currents in this range of membrane potentials are illustrated in Fig. 1B (arrowheads).

Isolation of single-channel currents in cell-attached patches of ROS 17/2.8 cells supports the notion that observed Ca²⁺ currents are generated by conduction through L-type channels. Single-channel currents were recorded in the cell-attached mode using 50Ba-EXT in the recording pipette as described under "Materials and Methods." Using a bath solution of 50Ca-EXT, a step depolarization exceeding 30 mV was required to elicit single-channel current events. With a depolarizing K-INT as the bath solution, this threshold was determined to be −10 mV. As shown in Fig. 2A, the frequency of events and the opening probability increased steeply with test potential while single-channel amplitude decreased. Single-channel slope conductance was determined to be 17.5 ± 0.4 pS (n = 6) for 50 mM Ba²⁺ over a range of potentials between −50 and +30 mV (Fig. 2B). The extrapolated reversal potential was greater than +60 mV, compatible with Ba²⁺ as the current-carrying cation. In Fig. 2C, opening probability was calculated from data like those shown in Fig. 2A, corrected for the number of channels apparent in the records at very positive test potentials. Over this range of potentials, these data could be described by a Boltzmann distribution with parameters similar to those capable of describing microscopic...
current: \[ P_0(V) = P_0(\text{max})[1 + \exp(V_m + V_0)/k]^{-1} \]
in which (i) \( P_0(\text{max}) = 0.8 \pm 0.12 \); (ii) \( V_0 = +11 \pm 4 \text{ mV} \); and (iii) \( k = 5 \pm 1 \text{ mV} \) \((n = 6)\). Open time distributions required description by two exponential components whose means were 2.2 and 16.4 ms \((\text{Fig. 2A})\), calculated for the data set at 0 mV as illustrated in Fig. 2A. Summed, averaged single-channel records showed activation and decay kinetics virtually identical to those of macroscopic currents \((\text{not shown})\). These results confirm the identity of this channel as the one responsible for generating the high threshold macroscopic current seen in these cells.

Another distinguishing feature of L-type Ca\(^{2+}\) channels is the sensitivity of current inactivation/decay rates to permeating divalent cations. Previous studies in cardiac and neuronal systems have shown that inactivation is increased upon elevation of intracellular Ca\(^{2+}\), thus decay rates are a function of current amplitude as well as voltage \((\text{for a review see Ref. 27})\). Ca\(^{2+}\) is the most effective and Ba\(^{2+}\) the least effective of the permeant ions in modulating inactivation. As shown in Fig. 3A, elevation of external Ca\(^{2+}\) from 5 to 20 mM increased the rate of inactivation. Current decay became biphasic, with a rapid initial transient component. Elevation of Ca\(^{2+}\) also shifted the activation voltage dependence 20 mV in the positive direction \((\text{Fig. 3B})\). Equimolar substitution of 20 mM Ba\(^{2+}\) for Ca\(^{2+}\) \((i)\) shifted activation back to more negative potentials, \((ii)\) reduced the initial transient component, and \((iii)\) increased peak current amplitude 2-3-fold \((\text{Fig. 3, A and B})\). Thus, both activation and inactivation of L-type channels in ROS 17/2.8 cells were strongly influenced by the type and concentration of external divalents.

A final distinguishing feature of L-type calcium channels is their sensitivity to the organic calcium channel drugs, of which the DHPs represent a major subclass. DHPs occur in both inhibitory and stimulatory forms and as a general rule can produce mixtures of agonist and antagonist effects that are concentration-dependent. Inhibitory DHPs, including nitrendipine, demonstrated mixed agonist and antagonist actions on Ca\(^{2+}\) currents in ROS 17/2.8 cells similar to those observed in cardiac muscle \((28)\). As demonstrated in Fig. 4A, lower concentrations of nitrendipine \((1 \mu M)\) exhibited stimulatory effects on Ca\(^{2+}\) currents that were elicited by depolarization from relatively negative holding potentials \((<-50 \text{ mV})\). These small stimulatory effects were most clearly observed at test potentials near threshold \((\text{i.e.} -10 \text{ mV})\). In contrast, higher concentrations of nitrendipine \((3 \mu M)\) reduced currents. Inhibitory effects were most prominent at positive test potentials \((\text{i.e.} +10 \text{ mV}, \text{Fig. 4A, lower traces})\). As shown in Fig. 4B, the DHP agonist BAY K 8644 increased the inward current elicited from a holding potential of -50 mV in a concentration-dependent manner. In contrast, at positive membrane potentials, the current was slightly reduced \((10-20\%)\) in the presence of BAY K 8644. At concentrations exceeding 5 mM, the inhibitory effects of BAY K 8644 began to predominate \((\text{Fig. 4C})\). This inhibition was accompanied by a progressive decrease in the rate of current inactivation. Additionally, in the presence of BAY K 8644, the threshold for activation was shifted from -20 to -40 mV, the net effect of which was to increase current severalfold at negative membrane potentials and concomitantly accelerate the activation rates \((\text{Fig. 4D})\).

1,25-(OH)\(_2\)-D\(_3\) and Other Vitamin D Metabolites Medulate L-type Current and Calcium Uptake in ROS 17/2.8 Cells—Experiments were performed to determine whether various metabolites of vitamin D, stimulated uptake of Ca\(^{2+}\) into cells from the external medium. As shown in Fig. 5, it was found that 1,25-(OH)\(_2\)-vitamin D\(_3\) preferentially stimulated uptake of Ca\(^{2+}\) by cultured osteosarcoma cells within 1 min.
**Fig. 2.** Single-channel currents in cell-attached patches. **A,** single-channel activity was recorded at the indicated test potentials using pipettes containing 50Ba-EXT. Bath solution contained K-INT to depolarize the cell to 0 mV. Holding potential: −50 mV. Superimposed openings at positive test potentials indicated that this patch contained at least four channels. **B,** single-channel current-voltage relation for cell-attached patches using pipettes containing 50Ba-EXT. Line through data points reflects a slope conductance of 17.5 pS. Data points indicated by arrows illustrate single-channel tail currents (generated by channel openings that persist beyond the cessation of the test depolarization, i.e. third and fifth trace at +10 mV in A). Points represent combined data from six patches; standard deviation lies within the span of the symbol. **C,** channel opening probability at several test potentials. Symbols show mean and standard deviation of data from four patches corrected for estimates of numbers of channels in each patch. Solid line was generated by a Boltzmann distribution whose parameters are given in the text. **D,** single-channel open time distribution for events evoked by depolarization to 0 mV. Solid line represents fit to a biexponential distribution with means of 2.2 and 16.4 ms (arrows). The amplitude of the component describing longer openings is 0.026 times that of the faster, thus 16% of the total open time is associated with the longer opening distribution.

**Fig. 3.** Calcium and barium currents exhibit inactivation properties dependent on ion concentration and composition. **A,** upper traces show effects of substitution of 20Ca-EXT for 5Ca-EXT. Inactivation rates acquire an initial transient component and current at later times is reduced. Lower trace shows the effect of subsequent substitution of 20Ba-EXT for 20Ca-EXT in the same cell. The initial transient component is proportionately reduced and current amplitude is increased approximately 3-fold. Holding potential: −50 mV. Test potential: +20 mV. **B,** peak current-voltage relations over a more extended range of test potentials in 5Ca-EXT (triangles), 20Ca-EXT (open circles), and 20Ba-EXT (filled circles). Note that activation threshold and maximum current are positively shifted by approximately 20 mV along the voltage axis by elevation of Ca²⁺. Equimolar substitution of Ba²⁺ for Ca²⁺ negatively shifts these parameters by approximately 15 mV.


**D_3 Modulates Calcium Current in Bone Cells**

Fig. 4. Dihydropyridines exhibit mixed agonist and antagonist effects on Ba^{2+} current. **A.** Upper traces show current elicited at a test potential of −10 mV in control (filled circle) and following addition of 1 μM (open circle) and 3 μM (open square) nitrendipine. Note that at this potential, an increase of current is observed at the lowest concentration. Lower traces show current elicited at a test potential of +10 mV from the same cell. Both concentrations of nitrendipine are inhibitory. Note the increase in current inactivation/decay rate at both concentrations and at both test potentials. Holding potential: −70 mV. **B.** Upper traces, current elicited at a test potential of −10 mV in control (filled circle), and following addition of 0.5 μM (open circle) and 1 μM BAY K 8644 (open square). Lower traces, current is increased slightly at a test potential of +10 mV by the lower concentration, but decreased to control levels by the higher one. **C.** Current from another cell showing the reduction of current at elevated BAY K 8644 concentrations. Filled circle, control; open circle, 1 nM; open square, 5 nM. Note the increase in current inactivation/decay rate at the higher concentration. Holding potential: −50 mV. Test potential: 0 mV. **D.** Peak current-voltage relations before (filled circles) and after (open circles) addition of 1 μM BAY K 8644. Note the shift in threshold and consequent increase in current at more negative test potentials.

Under the conditions of the assay (resting buffer, see "Materials and Methods") the uptake of Ca^{2+} was increased approximately 2-fold. The dose-response for this stimulatory effect was found to exhibit a maximum at concentrations between 0.5 and 1.0 nM with concentrations greater than 10 nM having essentially no measurable stimulatory effect. In contrast, other vitamin D_3 metabolites that we tested did not stimulate \(^{45}\text{Ca}^{2+}\) uptake at physiological concentrations. 25-(OH)_2-D_3 was found to stimulate uptake, but concentrations an order of magnitude higher than those of 1,25-(OH)_2-D_3 were required. 24,25-(OH)_2-D_3 did not stimulate uptake in the concentration range tested and indeed was slightly inhibitory at 5 nM.

Electrophysiological experiments were performed to test whether the effects of these vitamin D_3 metabolites on Ca^{2+} uptake were mediated by L-type Ca^{2+} channels. As seen in Fig. 6A, the addition of 5 nM 1,25-(OH)_2-D_3 to the external solution of patch clamped ROS 17/2.8 cells produced immediate changes in inward Ca^{2+} currents. The stimulation of current was increased further by reduction (by washout) of the D_3 to concentrations below 1 nM. Stimulation was dependent on the test potential, with maximal effects noted at negative test potentials and little if any stimulation recorded at positive test potentials (Fig. 6A). Interestingly, the effects of 1,25-(OH)_2-D_3 were qualitatively similar to those of the DHP BAY K 8644 (compare Fig. 6 and Fig. 4). In the presence of 1,25-(OH)_2-D_3, the threshold for activation of current is negatively shifted by approximately 20 mV and the inward current is increased at more negative test potentials (Fig. 6B). In addition, under depolarizing conditions (positive test potentials), 1,25-(OH)_2-D_3 was slightly inhibitory. As described above, these mixed agonist and antagonist effects are similar to those produced by DHPs.

Electrophysiological effects of other vitamin D_3 metabolites showed distinct quantitative differences from those of 1,25-
(OH)D$_3$. Little or no stimulatory effect could be observed at threshold test potentials (−10 mV) for concentrations of 24,25-(OH)$_2$-D$_3$ and 25-(OH)-D$_3$ of less than 5 nM (Fig. 7). Moreover, at more positive test potentials (+20 mV), a more pronounced inhibition of Ca$^{2+}$ current was observed. Stimulatory effects could be more clearly observed for both 24,25-(OH)$_2$-D$_3$ and 25-(OH)-D$_3$ at concentrations greater than 10 nM (data not shown).

**Modulation of Calcium Influx by 1,25-(OH)$_2$-Vitamin D$_3$ Is Sensitive to Membrane Depolarization**—Two additional types of calcium influx experiments were utilized to determine whether the effects of vitamin D$_3$ on ROS 17/2.8 cells could be regulated by membrane depolarization. 45Ca$^{2+}$ uptake by monolayers of ROS 17/2.8 cells was measured as described above and in Fig. 5. To test the effects of rapid membrane depolarization on 45Ca$^{2+}$ uptake, resting buffer was replaced by stimulating buffer containing high external K$^+$. As shown in Fig. 8, membrane depolarization alone was found to stimulate 45Ca$^{2+}$ uptake approximately 3-fold. This response is characteristic of cells possessing voltage-gated Ca$^{2+}$ channels. As shown above, addition of 0.5 nM 1,25-(OH)$_2$-D$_3$ was found to stimulate Ca$^{2+}$ uptake in resting buffer 1.6-fold relative to control. However, in stimulating buffer, no further increase in the rate of 45Ca$^{2+}$ could be elicited by the inclusion of 0.5 nM 1,25-(OH)$_2$-vitamin D$_3$. Indeed, a decrease to only 84% of control “stimulating” value was observed. These data were consistent with electrophysiological observations of vitamin D$_3$ action on ROS 17/2.8 cells which show slight inhibitory effects at depolarized test potentials (see Fig. 6).

**Modulation of Calcium Channels by 1,25-(OH)$_2$-Vitamin D$_3$**—To further investigate the mechanism of current stimulation by vitamin D$_3$, its effects were measured at the single channel level. As shown in Fig. 9A, addition of 3 nM 1,25-(OH)$_2$-D$_3$ to the bath solution of a cell attached patch induces prolongation of Ca$^{2+}$ channel open time during test depolarization to 0 mV. These consecutive data also illustrate an increased incidence of traces with lower opening probability than control. This phenomenon is more pronounced with more positive depolarization (data not shown) and underlies
Fig. 9. 1,25(OH)2-D3 prolongs open time of unitary Ca2+ channels in a manner analogous to Bay K 8644. A shows consecutive traces at a test potential of 0 mV, before (left) and after addition of 3 nM 1,25-(OH)2-D3. Note prolongation of channel openings and increased incidence of traces with low opening probability (3rd and 4th traces at right) relative to control. Holding potential: −50 mV. B illustrates that the increase in current reflected in summed, averaged single channel events elicited by vitamin D3 is quantitatively similar to that observed in macroscopic currents (compare with Fig. 6). C shows that the DHP agonist BAY K 8644 (1 μM) prolongs channel open time in a similar manner. Holding potential: −50 mV. Test potential: 0 mV. D quantitates increases in open times induced by 1,25-(OH)2-D3 in the same cell shown in A and B. In control (left), open times are fit by two exponentials with means of 1.2 and 9.2 ms. The relative (fit) amplitude of the longer distribution component is 0.006 times the faster. Following 1,25-(OH)2-D3 (right) open times are described by exponentials with means of 1.15 and 21.5 ms. The relative (fit) amplitude of the slower component is increased to 0.016.

DISCUSSION

Vitamin D3 has long been recognized as a calcitropic hormone that potentiates long term changes in bone structure and physiology. The biological activity mediated by interaction of vitamin D3 with intracellular steroid binding receptors that control gene expression in target cells has become generally recognized (for a review, see Ref. 29). ROS 17/2.8 cells respond to vitamin D3 and have been shown to possess significant amounts of the intracellular vitamin D3 receptor (30). The concentration of vitamin D3 required to induce transcriptional activation of genes associated with the osteoblast phenotype in bone cells is in the low nanomolar range (4, 31, 32). Physiological concentrations of vitamin D3 have also been reported to produce rapid changes in membrane permeability that are independent of gene expression (33, 34). The molecular mechanisms underlying this class of effect have remained poorly understood. In this report, we have demonstrated the presence of voltage-gated Ca2+ channels of the classical DHP-sensitive type in the osteoblast-like osteosarcoma cell line ROS 17/2.8. Furthermore, we have demonstrated that func-
tional properties of these channels are modulated by the steroid hormone 1,25-(OH)2-vitamin D3. This modulation is likely to be one of the primary mechanisms underlying hormone-mediated elevation of intracellular Ca2+ previously reported (16). Among the second messenger systems that may be activated by similar rises in Ca2+ levels are Ca2+/Ca2+-calmodulin-sensitive kinases, protein kinase C and phospholipase C (35). Likewise, activation of second messenger systems, including protein kinase C, by vitamin D3 might indirectly stimulate further Ca2+ influx via phosphorylation-dependent activation of L-type Ca2+ channels in the plasma membrane. The potential involvement of protein kinase C in Ca2+ channel activation in bone cells is supported by data using Fura 2-loaded cells (21).

In contrast to observations of Chesnay-Marchaise and Fritch (12) using mixtures of cells from neonatal rat calvaria, neither sodium current nor transient low threshold (T-type) Ca2+ current was detected in ROS 17/2.8 cells. Thus, regenerative electrical responses to depolarization (36, 37) are neither expected nor observed in this cell line. Nonetheless, it is clear that ROS 17/2.8 cells possess the repertoire of receptor molecules and ion channels necessary to mediate physiological responses to calcitropic hormones such as vitamin D3 or PTH.

The vitamin D3-responsive inward Ca2+ current in ROS 17/2.8 cells satisfies several criteria (namely its voltage dependence, single-channel properties, ion selectivity, and sensitivity to DHPs) used to define high threshold L-type Ca2+ channels in neurons, cardiac, and smooth muscle (13, 38-40). Single-channel measurements reveal a channel with 17.5 pS conductance to 50 mM Ba2+ whose open probability has voltage dependence appropriate to macroscopic high threshold current. Using the relation \( I = Np_o \) where \( I \) is macroscopic current, \( N \) is the number of functional channels/cell, \( p_o \) is opening probability, and \( i \) is single channel current, we estimate that ROS 17/2.8 cells possess a minimum of 1-2 x 106 L-type calcium channels/cell. By comparison, differentiated clonal BC3H1 myocytes express 1-2 x 104 channels/cell (41, 42), a value typical of "excitable" cells. It should be mentioned that because these experiments are not controlled with regard to phosphorylation state of the channels, a factor known to influence channel open times by stimulatory DHPs BAY K 8644 and 202-791 (48-50). These observations are supported by the data in Fig. 3. Contribution of Ca2+-activated outward currents is presumed to be minimal because linear leak-subtracted currents are inward even at very positive membrane potentials (> +60 mV) where outward current driving force is greatest. Taken together, this means that modulation of membrane surface potential (and thus channel-gating properties) by divalent cation concentration could operate as a negative feedback mechanism in an environment in which extracellular Ca2+ concentrations constantly fluctuate. It is precisely this type of dynamic cellular microenvironment that is predicted for bone cells participating in continuous remodeling of the calcified matrix (47).

Physiological concentrations of 1,25-(OH)2-vitamin D3 shift the voltage dependence of L-type channel activation in ROS 17/2.8 cells to more negative potentials (from -20 to -40 mV). Resting membrane potentials of osteoblasts and osteoblast-like cells range between -40 and -50 mV (36, 37). Thus, in physiological solutions, virtually no additional depolarization would be required to activate Ca2+ current in the presence of 1,25-(OH)2-D3. This is confirmed by the observation that 1,25-(OH)2-D3 consistently stimulated 45Ca2+ influx into resting, polarized ROS 17/2.8 cells.

1,25-(OH)2-D3 induces mixed agonist and antagonist effects on calcium current similar to those observed with the stimulatory DHPs BAY K 8644 and 202-791 (46-50). These competing pharmacological effects are strongly concentration-dependent; at lower concentrations stimulatory effects predominate whereas inhibitory effects increase at higher concentrations. Shifts in activation voltage dependence and prolongation of single-channel open times by stimulatory concentrations of 1,25-(OH)2-D3 or stimulatory DHPs could reflect stabilization of channel open states. The lack of strong holding potential dependence for inhibitory effects of 1,25-(OH)2-D3 suggests that binding to closed resting and inactivated states might be of similar affinity (51). Acceleration of decay/inactivation rates are not prominent at low, primarily stimulatory concentrations of 1,25-(OH)2-D3, similar to actions of DHP agonists like BAY K 8644. More elevated concentrations of 1,25-(OH)2-D3 and BAY K 8644 exhibit this inhibitory phenomenon. 24,25-(OH)2 and 25-OH-D3 metabolites produce more potent inhibitory effects on L-type current than does 1,25-(OH)2-D3. In preliminary single-channel recordings, these inhibitory properties are manifested as an increase in the number of nulls or failures of individual

\( D_3 \) Modulates Calcium Current in Bone Cells
channels to open during depolarization along with a reduced probability of reopening. Such actions are also observed with inhibitory DHPs (48) and have been interpreted as reflecting stabilization of the channel in the closed or inactivated states (51).

Although the functional similarity of vitamin D3 action to DHP modulation of channel activity is quite striking, it must be emphasized that there is no direct evidence that vitamin D3 binds specifically to DHP-sensitive Ca2+ channels. Several models have been proposed to explain the competing stimulatory and inhibitory properties of the DHPs which might ultimately be useful in evaluating vitamin D3 action. Pharmacological and electrophysiological studies suggest that there may be at least two distinct stereoselective DHP-binding sites on the Ca2+ channel complex whose occupation by ligand is associated with agonist and antagonistic effects (28, 49). Other models propose DHPs bind to a single site in the channel that has different affinities for DHPs in the resting, open, or inactivated channel states (50). Vitamin D3 does not bear any obvious structural similarity to the DHPs or to other Ca2+ channel ligands, therefore, it is difficult to assume that it binds to previously characterized regulatory sites on the Ca2+ channel protein. If vitamin D3 does interact directly with channel protein(s), it might be expected to modulate DHP-sensitive Ca2+ channel activity in other cell types. 1,25-(OH)2D3 has been shown to increase DHP-sensitive Ca2+ uptake in primary skeletal muscle cell cultures (52). Preliminary experiments in our laboratory indicate that 1,25-(OH)2D3 increases Ca2+ influx and DHP-sensitive Ca2+ current in both BC3H1 and C2 clonal myocytes, which possess the skeletal muscle channel isotype. It is also possible that the rapid effects of vitamin D3 metabolites are mediated indirectly by activation of second messenger systems. This notion is supported by previous studies (21) implicating protein kinase C activation in Ca2+ influx in bone cells. Further studies are necessary to distinguish the alternatives of direct versus indirect action of vitamin D3 on voltage-sensitive Ca2+ channels.

The association between vitamin D3 deficiency, aberrant calcium metabolism, and calcifying bone disorders such as rickets has long been known (for a review, see Ref. 53). Likewise, deficiencies in vitamin D3 can result in muscle weakness and alteration in contractility (reviewed in Ref. 54). Conversely, systemic administration of supraphysiological doses of vitamin D3 or dihydrotriacysteol can induce severe myopathies in both smooth and cardiac muscle with accompanying calcinosis of major blood vessels (55, 56). These phenomena are associated with tissue Ca2+ overload that have been previously attributed to effects of vitamin D3 on genetic regulatory mechanisms. It is of interest that these symptoms can be circumvented experimentally by both organic and inorganic Ca2+ channel blockers (55). In light of recent findings including these presented here, it seems clear that changes in Ca2+ channel function induced by abnormal concentrations of vitamin D3 metabolites may also contribute to these disorders in D3-responsive tissues including muscle and bone.

Acknowledgments—We would like to thank Drs. William T. Butler, Daniel Carson, John Dani, Glenn Decker, Renny Franceschi, and Tony Lacerda for their helpful comments regarding this manuscript. Karen Stewart is gratefully acknowledged for her assistance in the preparation of the text.

1 J. M. Caffrey and M. C. Farach-Carson, unpublished observations.

REFERENCES

D₃ Modulates Calcium Current in Bone Cells