Stimulation by 1α,25(OH)₂-Vitamin D₃ of Whole Cell Chloride Currents in Osteoblastic ROS 17/2.8 Cells

A STRUCTURE-FUNCTION STUDY*

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EXPERIMENTAL PROCEDURES

Cell Culture—ROS 17/2.8 cells (obtained from M. C. Farach-Carson) were cultured in Ham's F-12 medium (Sigma) containing 5% fetal bovine serum (Sigma) and 5% Serum Plus (JRH Biosciences, Woodland, CA), as described previously (11). For patch-clamp experiments, cells were plated at very low density in 35-mm tissue culture dishes. Prior to recordings, the cells were washed at least three times with the electrophysiological external solution to remove the medium completely.

Electrophysiology—Initially we used recording solutions designed by others (9). The composition of the external (extracellular) solution was: 110 mM N-methyl-D-glucamine, 20 mM BaCl₂, 130 mM glutamate, 10 mM Hepes, and 20 mM glucose (pH 7.4, adjusted with N-methyl-D-glucamine). The pipette (intracellular) solution contained: 140 mM tetraethylammonium-Cl, 0.5 mM MgCl₂, 1.3 mM CaCl₂, 20 mM BaCl₂, and 10 mM Hepes (pH 7.4, adjusted with tetraethylammonium-OH). The corresponding pipette solution contained 140 mM CsCl, 1.2 mM MgCl₂, 1 mM EGTA, and 10 mM Hepes (pH 7.4, adjusted with CsOH). The osmolality of the solutions was 290 mosmol/kg (adjusted with glucose). The presence of 20 mM external BaCl₂ allowed the simultaneous recording of inward currents through Ca²⁺ channels. A high concentration of tetraethylammonium⁺ outside and Ca²⁺ inside the cell prevented the recording of K⁺ channel activity.

Patch-clamp recordings were performed with an Axopatch 1C patch-clamp amplifier (Axon Instruments, Foster City, CA). Patch pipettes were fabricated from Drummond capillaries (Drummond Scien-
The structures of 1α,25(OH)2D3 and its analogs. 1α,25(OH)2D3, 25(OH)2D3, and 25(OH)2D3 are natural metabolites of the parent vitamin D3; these compounds are classified as secosteroids as a consequence of the broken 9,10-carbon bond (compare with analog JN). Each of the secosteroids (1α,25(OH)2D3, 25(OH)2D3, and 1α,25(OH)2D3) are able to generate a continuum of conformational shapes due to rotation around the 6,7-carbon bond; this is illustrated in the top panel for 1α,25(OH)2D3. In the limit there may be either the 6-s-trans (steroid-like) conformer or the 6-s-cis (steroid-like) conformer. 1α,25(OH)2D3 (analog HL), 1α,25(OH)2-tachysterol (analog JN), and 1α,25(OH)2-tachysterol (analog JB) are synthetic analogs. Analogs JN and JB are 6-s-cis and 6-s-trans locked conformers, respectively. 1α,25(OH)2D3 has been reported in other studies to be an antagonist of the 1α,25(OH)2D3, rapid responses of transcalcitria (30) and Ca2+ uptake in ROS 17/2.8 cells (31).

RESULTS

Effects of 1α,25(OH)2D3 on Ion Currents in ROS 17/2.8 Cells—The structures of the conformationally flexible 1α,25(OH)2D3 and other analogs employed in this study are shown in Fig. 1.

We first recorded the activity of previously described voltage-dependent L-type Ca2+ channels in the ROS 17/2.8 cells (9, 10, 15). Fig. 2 shows the effect mediated by 1α,25(OH)2D3 on Ba2+ currents when it was added to the bath at a final concentration of 0.5 nM. This effect, which have been described before (9, 10, 15), was characterized by a drastic shift of IV relations of about 25 mV to more negative potentials in the case of this particular cell and developed over the course of the first few minutes after the addition of the hormone. In this experiment, an increase in the concentration of 1α,25(OH)2D3 to 5 nM did not cause any additional effect, although this result varied among different cells. We recorded a mean shift of −10.4 ± 2.6 mV in 8 out of 22 cells (36%) studied under the same conditions. These 1α,25(OH)2D3-sensitive inward Ba2+ currents were almost completely blocked by 100 μM Cd2+, a Ca2+ channel blocker, subsequently added to the bath. As postulated previously (9), this modification of the voltage sensitivity of Ca2+ channels in osteoblasts by 1α,25(OH)2D3 may result in the facilitation of Ca2+ channel opening by the hormone for Ca2+ uptake at membrane potentials close to the resting value, which has been
reported to be in the range of $-10$ to $-40$ mV in osteoblasts (25, 28, 29).

In a preliminary communication we reported that 1α,25(OH)₂D₃ also affects Cl⁻ channel activity in the ROS 17/2.8 cells (11). In the presence of 100 μM Cd²⁺ in the bath which effectively blocks Ca²⁺ channel activity, an outward current was recorded in the range of $-30$ to 80 mV in the glutamate-containing external solution (see “Experimental Procedures”) in approximately 80% of the cells, as shown in Fig. 3. The addition of a final concentration of 0.05 nM 1α,25-(OH)₂D₃ to the bath caused, in the case of this particular cell, a 1.2-fold increase of the outward current measured at 80 mV over the course of 2.5 min. The time course of this effect is shown in Fig. 3B. After a lag period of about 45 s, which could be attributed to the time required for diffusion of the steroid to the cell and/or the stimulation of cellular signal transduction pathways, the rapid increase of the current took place over the course of the next 30 s. 1α,25(OH)₂D₃ promoted the increase of outward currents in 14 out of 15 cells (93%) studied under the same recording conditions. This enhancing effect was dependent on the concentration of the hormone in the range of 0.05–50 nM (see also Fig. 5).

Permeability studies carried out by replacing the main anion in the recording solution are summarized in Table I. We found that the 1α,25(OH)₂D₃-sensitive outward current was permeable to glutamate and Cl⁻, suggesting a poor anion discrimination of this channel. On the other hand, gluconate in the external solution significantly decreased outward currents, as expected for a less permeable anion. The addition of 200 μM DIDS, a specific Cl⁻ channel blocker, to the bath blocked the 1α,25(OH)₂D₃-enhanced outward currents, as shown in Fig. 4. This blockade by DIDS was time- and voltage-dependent. It developed over the course of 1–2 min after the addition of the agent to the bath. As described before for the blockade by DIDS of the cAMP-activated Cl⁻ currents in primary cultured rat osteoblasts (22) and of the mechanosensitive Cl⁻ channels in ROS 17/2.8 cells (23), these 1α,25(OH)₂D₃-sensitive Cl⁻ currents were strongly reduced by the agent but were not completely blocked. DIDS was shown to be effective at a concentration of 200 μM when any of the anions shown in Table I were used.

Fig. 4 also shows the outward rectification of these Cl⁻ currents. No inward currents were recorded at membrane potentials below the reversal potential ($E_{rev}$) for Cl⁻ in the Cl⁻-containing solutions (see “Experimental Procedures”). The $E_{rev}$ for Cl⁻ calculated from the Nernst equation gave a value of $\sim$–6 mV, which is very close to the value found from the recordings.

**Antagonist Effects of the 1-Hydroxy Epimer 1β,25(OH)₂D₃—**

The synthetic analog 1α,25(OH)₂D₃ (analog HL), which only differs from the natural metabolite in the orientation of the hydroxy group on carbon 1 (see Fig. 1), has been shown by this laboratory to inhibit the rapid activation of transepithelial calcium transport by 1α,25(OH)₂D₃ in chick intestine (30) and Ca²⁺ uptake in ROS 17/2.8 cells (31). Analog HL has also been shown to reduce the agonist potency by 1α,25(OH)₂D₃ of Ca²⁺ channels in ROS 17/2.8 cells (32). In this work, we investigated the effects of 1β,25(OH)₂D₃ on outward Cl⁻ currents in the osteoblastic cells. No enhancement of the Cl⁻ currents by 1 nm 1β,25(OH)₂D₃ was found in 44% of the studied cells, while we recorded a modest 1.1–1.3-fold increase of the outward currents at 80 mV in the remaining 56% of the cases (9 of 16 cells) (data not shown). The addition of 0.05–5 nm 1α,25(OH)₂D₃ to the bath in the presence of 1 nm HL promoted a significantly lower increase of the outward anion current if

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**TABLE I**

Permeability of the 1α,25(OH)₂D₃-sensitive outward conductance in ROS 17/2.8 cells to different anions

| External solution | Main anion | Main cation | Internal solution | Main anion | Main cation | Current amplitude (pA) |
|-------------------|------------|-------------|-------------------|------------|-------------|-----------------------|
| 130 glutamate     | 110 NMDG⁺  | 150 Hepes   | 100 NMDG⁺         | 178 ± 19 (n = 9) |
| 140 Cl⁻           | 140 TEA⁺   | 140 Cl⁻     | 140 Ca⁺           | 486 ± 106 (n = 14) |
| 140 gluconate     | 140 Na⁺    | 140 Cl⁻     | 140 Ca⁺           | 5 ± 3 (n = 5) |

The outward current amplitude was measured at 80 mV. Concentrations of ions are in mM. NMDG, N-methyl-D-glucamine; TEA, tetraethylammonium.
ing traces of currents elicited by a depolarizing step to 60 mV, obtained at 80 mV during the first minute after the addition of 5 nM 1a,25(OH)2D3. The magnitude of outward currents was partially blocked by the subsequent addition of 200 μM DIDS. The remaining dihydropyridine-insensitive outward current showed a 2-fold increase of the amplitude at 80 mV during the first minute after the addition of 5 nM 1α,25(OH)2D3 (open circles). Finally, the 1α,25(OH)2D3-promoted outward currents were partially blocked by the subsequent addition of 200 μM DIDS, a stilbene derivative specific for Cl− channels (inverse open triangles). Currents were activated by 100-ms duration depolarizing voltage steps to between −60 and 80 mV. The numbers in parentheses indicate the sequential order of the recordings. B shows the corresponding traces of currents elicited by a depolarizing step to 60 mV, obtained after the addition of each agent. The holding potential was −70 mV.

compared with the increments recorded for the hormone alone (see Fig. 5). On the contrary, when the concentration of 1α,25(OH)2D3 was raised to 50 nM, the increase of the outward currents in the presence of the β-stereoisomer was similar to that measured for the hormone alone, suggesting that both ligands may be competing for the same receptor. These results with HL suggest that the 1-hydroxy epimer 1β,25(OH)2D3 may act as an antagonist of the 1α,25(OH)2D3 effects on Cl− channels in osteoblasts as it blocked the effects of the hormone on these cells.

Effects of the Natural Metabolite 25-OH-D3—The natural metabolite 25-OH-D3 promoted an increase in Cl− outward currents in 12 out of 14 (86%) ROS 17/2.8 cells within the first 5 min when added to the external solution at a concentration of 0.05–5 nM. In the case of the cell shown in Fig. 6A, 5 nM 25-OH-D3 caused a 2.7-fold increase of the current at 80 mV. On the other hand, 25-OH-D3 did not cause any significant modification of IV relations for inward Ba2+ currents in the range of 0.05–50 nM (see Fig. 6B), which agrees with previously published results (9, 10). The specificity of this effect of 25-OH-D3 on Cl− currents, which was not found on Ba2+ currents in the same cellular system, may be indicative of different modulatory mechanisms underlying the mode of action of the vitamin D metabolites.

Enhancement of Cl− Currents by a 6-s-cis Locked and a 6-s-trans Locked Analog—It has been recently shown that synthetic vitamin D analogs locked in the 6-s-cis position (steroid-like molecules, see Fig. 1) are potent agonists for the rapid effects of the hormone, while 6-s-trans locked conformers (extended forms) are inactive for the same responses in target cells (27, 33). Noticeable transcalcitria-promoting effects in chick intestinal epithelium and 45Ca2+ uptake by ROS 17/2.8 cells have been described specifically for the synthetic con-
former 1α,25(OH)₂-lumisterol₃ (analog JN), which is locked in the 6-s-cis position (27). We found that 1–10 nM analog JN caused a 2.2 ± 0.7-fold increase of the outward anion conductance in 7 out of 10 ROS 17/2.8 cells (70%). This response did not differ significantly from the effects promoted by 0.5 nM 1α,25(OH)₂D₃ (p < 0.05, see Fig. 7). On the other hand, 1α,25(OH)₂-tachysterol (analog JB), a synthetic 6-s-trans conformer which has been shown to be inactive in both transchaltachia and ⁴⁵Ca²⁺ influx in osteoblasts (27) promoted only a modest 0.8 ± 0.3-fold increase of Cl⁻ currents at 80 mV when applied at 1–10 nM in 80% of the cells. In this case, the response differed significantly from the enhancing effect exerted by 0.5 nM 1α,25(OH)₂D₃ (p < 0.05, Fig. 7).

The magnitude of the effects on Cl⁻ currents promoted by the different analogs of 1α,25(OH)₂D₃ used in this work is shown comparatively in Fig. 7. Note that the effects promoted by 1–10 nM JN and 1–10 nM JB on these Cl⁻ currents differed significantly from each other (p < 0.01).

### Specificity of the Response by 1α,25(OH)₂D₃ Analogs—

The specificity of ion channel responses to 1α,25(OH)₂D₃ analogs was investigated by means of the evaluation of possible effects exerted by other steroids on the Cl⁻ outward currents in ROS 17/2.8 cells. Steroid hormones have been demonstrated to modify ion channel activity in different cells (34–36). As also shown in Fig. 7, 50 nM cholesterol and 0.01–10 nM β-estradiol did not have any significant effect on the Cl⁻ currents studied in this work.

### DISCUSSION

It has been demonstrated in osteoblasts that different hormones taking part in the process of bone remodeling affect ion channel activity in the plasma membrane. More specifically, electrophysiological measurements carried out on the bone-forming cells have shown that the seco steroid 1α,25(OH)₂D₃ increases Ca²⁺ influx through voltage-activated Ca²⁺ channels by facilitating the opening of the channels at membrane potentials close to the resting value (9, 10) and also enhances an outward K⁺ current activated by depolarization (37). However, the precise mechanisms for ion channel modulation by the hormone and their physiological role remain unknown.

We recently described in a preliminary report the enhancing effect of 1α,25(OH)₂D₃ on Cl⁻ currents in the osteoblastic cell line ROS 17/2.8 cells (11). In the present work, we describe in more detail the effects of 1α,25(OH)₂D₃ and related structural analogs on these currents, this being the first extended report on a Cl⁻ conductive membrane pathway involved in the rapid responses of this seco steroid.

We found an outwardly rectifying voltage-dependent Cl⁻ current activated upon depolarization in ROS 17/2.8 cells that can be increased by external application of physiological concentrations of the hormonally active 1α,25(OH)₂D₃. This effect was found in 93% of the studied cells. Since patch-clamp currents are defined on the basis of the direction of the movement of positive charges, an outward current in the case of anions represents an influx of the negative charges into the cell. Therefore, according to our results, the depolarization of the osteoblast membrane from the resting value (~40 to ~10 mV, according to Refs. 28 and 29) activates an influx of anions, Cl⁻ being the most abundant one under physiological conditions.

The potentiation of the anion currents by 1α,25(OH)₂D₃ was dependent upon the concentration of the hormone and had a maximal increase at 0.5–5 nM followed by an attenuation at 50 nM. A biphasic effect of 1α,25(OH)₂D₃ was also previously described for other nongenomic effects of the hormone including the promotion of Ca²⁺ uptake in ROS 17/2.8 cells and the rapid Ca²⁺-transport process in the chick intestinal epithelium (transchaltachia) (9, 33).

The enhancing effect of 1α,25(OH)₂D₃ on the anion currents in the osteoblastic cell line developed rapidly, in the course of only a few seconds to minutes. The rapidity of these effects suggests that they are different from the classical steroid receptor-mediated nuclear effects and are part of an increasing list of 1α,25(OH)₂D₃-mediated nongenomic effects described in different target cells (2, 3, 30, 33, 38–41).

Structure-function studies carried out with different structurally related 1α,25(OH)₂D₃ analogs on different target cellular systems have proved to be very useful in defining the molecular steps involved in the mechanisms of action of the hormone (reviewed in Ref. 1). In the case of rapid nongenomic effects postulated to be initiated at the plasma membrane level, the use of synthetic analogs has led to the discovery that some structural forms are “preferred” over others which have been shown to be more effective in genomic processes. The natural seco steroid 1α,25(OH)₂D₃ exists as a continuum of potential shapes extended from the 6-s-cis (steroid-like) to the 6-s-trans (extended, see Fig. 1) which may interact with the receptors. It has been shown recently that synthetic 6-s-trans locked analogs are inactive in both rapid and some genomic responses, while 6-s-cis locked analogs are potent agonists of membrane-initiated nongenomic effects (27). In the present work, we demonstrate for the first time that the 6-s-cis locked analog 1α,25(OH)₂-lumisterol₃ significantly increased outwardly rectifying voltage-activated Cl⁻ currents in ROS 17/2.8 cells, whereas the enhancing effects by 1α,25(OH)₂-tachysterol, the corresponding 6-s-trans conformer, were significantly lower (see Fig. 7).

In a related study the 1β epimer of 1α,25(OH)₂D₃, 1β,25(OH)₂D₃, which has been shown to block the rapid effects of the hormone (30–32) and to bind to the cellular membrane in osteoblasts (42), remarkably decreased the potentiation of Cl⁻ currents by 1α,25(OH)₂D₃ in ROS 17/2.8 cells (see Fig. 5), suggesting that it may also act as an antagonist of ion channel responses by the hormone. We conclude that inversion of the orientation of the hydroxyl on carbon 1 of the hormone (1β for 1α) may be enough to block the response of cell ion channels by competitively binding to the same surface receptor as a first
step in the process.

Finally, the specific effects found for the natural metabolite 25-OH-D₃ on Cl⁻ currents but not on Ba²⁺ currents in ROS 17/2.8 cells (see Fig. 6) open the possibility to the existence of different cellular modulatory mechanisms underlying the control of membrane ionic pathways and the electrical state of the cell by active 1α,25(OH)₂D₃.

Although there is growing evidence that 1α,25(OH)₂D₃ may exert its rapid, nongenomic effects by interacting with a putative mVDR and by initiating a series of molecular pathways involving second messengers, future experiments need to be carried out to elucidate the molecular steps linking the hormonal signal and the specific enhancement of voltage-dependent outward Cl⁻ currents in osteoblasts.

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