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Comparison of the Relative Effects of 1,24-Dihydroxyvitamin D_2 [1,24-$(OH)_2D_2$], 1,24-Dihydroxyvitamin D_3 [1,24-$(OH)_2D_3$], and 1,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] on Selected Vitamin D-Regulated Events in the Rat

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ABSTRACT. The present experiments were conducted to compare the relative hypercalciuric and hypercalcemic activities of 1,24-dihydroxyvitamin D_2 [1,24-$(OH)_2D_2$], 1,24-dihydroxyvitamin D_3 [1,24-$(OH)_2D_3$], and 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] in 7-week-old rats. The rats were dosed orally with each sterol for 7 days at a rate of 1 ng/g body weight/day. We also monitored the effect of the three compounds on the induction of mRNA for CaATPase and for 25-hydroxyvitamin D-24-hydroxylase in the kidney and intestine, on plasma vitamin D metabolite levels, and on the capacity to evoke modification in the vitamin D receptor/retinoic acid X receptor (VDR/RXR) heterodimer conformation. Plasma calcium was elevated in the rats treated with 1,24-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$, but not in the 1,24-$(OH)_2D_2$-dosed rats. Urinary calcium was elevated significantly (relative to controls) in all groups. The order of hypercalciuric activity was 1,25-$(OH)_2D_3$ $\geq$ 1,24-$(OH)_2D_3$ $\geq$ 1,24-$(OH)_2D_2$ $>$ control. Duodenal plasma membrane calcium ATPase (PMCA) mRNA was elevated to a similar extent in all groups relative to controls. Duodenal 24-hydroxylase mRNA was elevated in all groups relative to controls; however, the elevations were significantly higher in the 1,24-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ groups compared with the 1,24-$(OH)_2D_2$ group. Kidney 24-hydroxylase also was elevated significantly in the 1,24-$(OH)_2D_3$- and 1,25-$(OH)_2D_3$-treated rats but not in the 1,24-$(OH)_2D_2$-treated rats. Recombinant human vitamin D receptor (hVDR) extracts were incubated with saturating concentrations of 1,24-$(OH)_2D_2$, 1,24-$(OH)_2D_3$, and 1,25-$(OH)_2D_3$ and subsequently analyzed by electrophoretic mobility shift assay (EMSA). Overall binding was comparable for all metabolites; however, the 1,24-$(OH)_2D_2$ complex exhibited distinctly altered mobility relative to 1,24-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$, suggestive of an effect on hVDR/hRXR conformation. These data suggest that 1,24-$(OH)_2D_2$ is not as potent as either of the vitamin D_3 sterols at affecting hypercalcemia or hypercalciuria in young growing rats; however, 1,24-$(OH)_2D_2$ can evoke other biological responses similar to the vitamin D_3 sterols. These different responses may be related to the alterations in conformation state of the hVDR/hRXR heterodimer. BIOCHEM PHARMACOL **60**;5:701–708, 2000.

KEY WORDS. 1; 24-dihydroxyvitamin D_2; 1; 25-dihydroxyvitamin D_3; hypercalcemia; hypercalciuria

The 24-position of vitamin D_2, in contrast to the similar position in vitamin D_3, can be considered to be highly reactive. It is both a tertiary carbon and an allylic position, and the formation of a reactive intermediate (radical, cation) at this position would be highly stabilized. The proximity of this reactive center to the 25-position, therefore, would afford the possibility of C-24-hydroxylation of vitamin D_2. Jones *et al.* [1] demonstrated that this is indeed the case when they isolated 24-OH-D_2¶ from plasma of male rats treated with 100 IU of radiolabeled vitamin D_2.

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¶ *Abbreviations:* 24-OH-D_2, 24-hydroxyvitamin D_2; 25-OH-D_2, 25-hydroxyvitamin D_2; 1,24-$(OH)_2D_2$, 1,24-dihydroxyvitamin D_2; 1,24-$(OH)_2D_3$, 1,24-dihydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 1,25-$(OH)_2D_2$, 1,25-dihydroxyvitamin D_2; 1α-OH-D_2, 1α-hydroxyvitamin D_2; 1α-OH-D_3, 1α-hydroxyvitamin D_3; 1,24,25-$(OH)_3D_3$, 1,24,25-trihydroxyvitamin D_3; 1,24,25-$(OH)_3D_2$, 1,24,25-trihydroxyvitamin D_2; 1,24,26-$(OH)_3D_2$, 1,24,26-trihydroxyvitamin D_2; VDRE, vitamin D response elements; 24-hydroxylase, vitamin D-24-hydroxylase; VDR, vitamin D receptor; rhVDR, recombinant human vitamin D receptor; RXR, retinoic acid X receptor; rhRXR, recombinant human retinoic acid X receptor; DTT, dithiothreitol; RT–PCR, reverse transcriptase–polymerase chain reaction; EMSA, electrophoretic mobility shift assay; RIA, radioimmunoassay; and $PMCA_1$ plasma membrane calcium ATPase isoform 1.

Engstrom and Koszewski [2] extended these observations by demonstrating that the production of 24-OH-D_2 exceeds formation of 25-OH-D_2 by pig liver extracts *in vitro*. In experiments with rats, Horst *et al.* [3] showed that the concentration of 24-OH-D_2 in plasma is ~20% that of 25-OH-D_2 in rats receiving physiologic doses (100 IU/day) of vitamin D_2 and is equivalent to 25-OH-D_2 in rats receiving superphysiologic (800 IU/day) doses of vitamin D_2. Furthermore, 24-hydroxylation has been shown to be an activation pathway for vitamin D_2 with the demonstration that 24-OH-D_2 stimulates intestinal calcium transport to an extent similar to 25-OH-D_2 and that 24-OH-D_2 can be 1α-hydroxylated to form 1,24-$(OH)_2D_2$, which binds to VDR with a slightly lower affinity than that of 1,25-$(OH)_2D_2$ and 1,25-$(OH)_2D_3$ [3].

In addition to representing a minor, but significant, activation pathway for vitamin D_2 metabolism, the formation of 1,24-$(OH)_2D_2$ also represents a pathway for the activation of 1α-OH-D_2. Studies using human hepatoma cells have shown that increasing the concentration of 1α-OH-D_2 in the medium from 1 nM to 10 μM produces a disproportionate increase in 24-hydroxylation compared with 25-hydroxylation, so that 1,24-$(OH)_2D_2$ predominates at higher substrate concentrations (>100 nM) [4].

Although 24-hydroxylation appears to be a significant physiologic pathway for vitamin D_2 metabolism, the analogous pathway for vitamin D_3 has never been convincingly demonstrated to occur *in vivo*. Furthermore, using the human hepatoma cell cultures, Strugnell *et al.* [4] could not demonstrate the production of 1,24-$(OH)_2D_3$ when 1α-OH-D_3 was used as substrate.

Nonetheless, 1,24-$(OH)_2D_3$ has been chemically synthesized, and its biologic activities have been evaluated. Reports suggest that both 1,24-$(OH)_2D_2$ and 1,24-$(OH)_2D_3$ are active *in vivo* but may be less hypercalcemic than 1,25-$(OH)_2D_3$ [3, 5–9]. The present experiments extend these studies to compare the calcemic effects of these two analogs and to evaluate the *in vivo* responsiveness of vitamin D-regulated genes to 1,24-$(OH)_2D_2$, 1,24-$(OH)_2D_3$, and 1,25-$(OH)_2D_3$ in vitamin D_3-replete rats.

MATERIALS AND METHODS

Vitamin D Compounds

(24*S*)-1,24-$(OH)_2D_2$ was provided by Bone Care International, (24*R*)-1,24-$(OH)_2D_3$ was a gift from Dr. Hector DeLuca, and 1,25-$(OH)_2D_3$ was a gift from Dr. Milan Uskokovic. The compounds were dissolved in 50 μL ethanol, which was diluted further with fractionated coconut oil to give a final concentration of 2 ng/μL.

Rats

Male weanling rats (3 weeks of age) were purchased from Sprague–Dawley and maintained on a commercial diet purchased from Purina Mills, Inc. containing 1.01% calcium and 0.74% phosphorus. The rats were housed in hanging wire cages until they reached a weight of ~200 g (7–10 days), after which they were transferred to individual metabolic cages. The rats were allowed an additional 7 days of adaptation prior to beginning a 7-day dosing and collection period. During the daily dosing and collection period, rats received vitamin D compounds orally at the rate of 1 ng/g body weight. Urine was collected daily into 50-mL polyethylene tubes containing 0.2 mL acetic acid. Each morning the urine tubes were removed from cages and frozen immediately. During the adaptation and collection periods, metabolic cages were disassembled and cleaned on a daily basis to assure that urine and feces were freely flowing and to prevent the potential contamination of excretory products with feed. At the end of the 7-day collection period, the rats were decapitated (under CO_2:O_2 1:1 anesthesia) 4–5 hr after receiving their final dose of analogue. Blood and tissues were collected for assays.

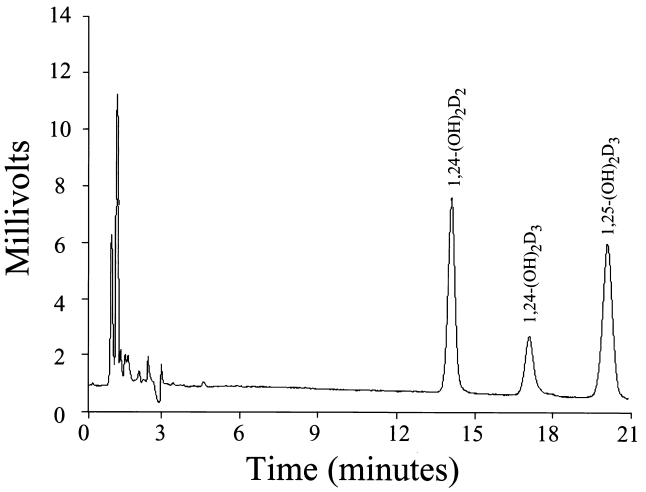

FIG. 1. Elution of 1,24-$(OH)_2D_2$, 1,24-$(OH)_2D_3$, and 1,25-$(OH)_2D_3$ from an Econosphere 3-μm silica column developed in hexane:isopropanol:methanol (96:2.6:1.4) at a flow rate of 2.0 mL/min.

Plasma and Urine Assays

Isolation of the dihydroxyvitamin D metabolites was achieved using the C_{18}OH extraction system [10]. In this system 1,24-$(OH)_2D_2$ and 1,24-$(OH)_2D_3$ co-migrate with 1,25-$(OH)_2D_3$ (data not shown). Therefore, [^{3}H]-1,25-$(OH)_2D_3$ was used for recovery estimates. The three vitamin D sterols were separated by HPLC using an Econosphere 3-μm silica column developed in hexane: isopropanol:methanol (96:2.6:1.4) (Fig. 1). The fractions containing the vitamin D sterols were collected individually and assayed using analytical reagents from Diasorin, Inc. The polyclonal antibody used had equal affinity for the 1α-hydroxylated metabolites (Fig. 2). Therefore, 1,25-$(OH)_2D_3$ was used as the RIA standard for estimating the concentrations of each of the three sterols. Plasma and urine calcium were measured by atomic absorption spectrophotometry. Urinary creatinine was measured colorimetrically [11].

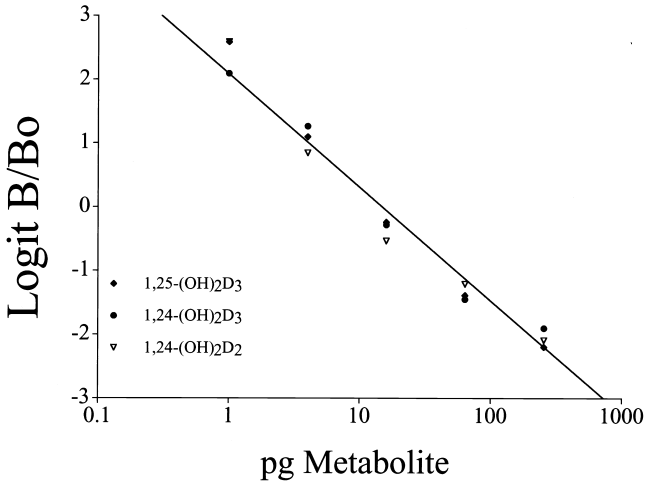

FIG. 2. Displacement potency of 1,24-$(OH)_2D_2$, 1,24-$(OH)_2D_3$, and 1,25-$(OH)_2D_3$ in the RIA used for assaying plasma concentrations of these vitamin D sterols.

Kidney and Duodenal $PMCA_1$ and 24-Hydroxylase Oligonucleotide Primers for RT–PCR

The duodenal mucosa and kidneys were collected, frozen immediately in liquid nitrogen, and stored at −80°. Total RNA from frozen tissues was isolated by using Trizol reagent and a modified single-step RNA isolation method developed by Chomczynski and Sacchi [12].

Oligonucleotide primers for $PMCA_1$ were 5′-GCC ATC TTC TGC ACA ATT GT-3′ (sense primer), nt 3200–3219, and 5′-TCA GAG TGA TGT TTC CAA AC-3′ (antisense primer), nt 3825–3844. Oligonucleotide primers for 24-hydroxylase were 5′-ACC GCC TAC AGA GAT ATT CCC AG-3′ (sense primer), nt 1206–1228, and 5′-ACC CGA AAC CGT TGG TTT GTC-3′ (antisense primer), nt 1558–1578. All oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc.

The mimics for $PMCA_1$ and 24-hydroxylase were prepared as follows. The $PMCA_1$ mimic DNA fragment is a homologous sequence to $hPMCA_1$ cDNA. The 644-bp $PMCA_1$ band resulting from the RT–PCR using the primers described above was digested with *Dde*I. The internal sequence, 156 bp, was removed; the flanking sequences were ligated, and the resulting 488-bp mimic was amplified using the $PMCA_1$ primer set. This DNA was used as the mimic for $PMCA_1$ analysis. The 24-hydroxylase standard DNA fragment was a heterologous sequence to 24-hydroxylase cDNA. It was prepared using the Clontech mimic construction kit (Clontech), according to the manufacturer's instructions. This resulted in a 244-bp mimic DNA that was amplified by the 24-hydroxylase primers.

The RT–PCR was set up in a single-tube reaction. Briefly, 0.25 or 0.5 μg of total RNA was used to quantitate the mRNA of $PMCA_1$ or 24-hydroxylase, respectively. The reaction was adjusted with the following conditions in a total volume of 50 μL: 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.8 mM of each dNTP, 50 or 25 pmol oligonucleotide primers ($PMCA_1$ or 24-hydroxylase primers, respectively), 10 U RNasin (Promega), 25 U Moloney murine leukemia virus (M-MLV) RT (Gibco/BRL), 1.25 U *Taq* DNA polymerase (Boehringer), 0.3 μCi [α-^{33}P]-dCTP (specific activity 1000–3000 Ci/mmol). Standard DNA fragments were added in increasing amounts (0.0313 to 0.4 pg) to reaction tubes. The RT reaction was performed at 56° for 40 min, and finally at 95° for 4 min, to terminate the M-MLV RT activity. The PCR was conducted in a Perkin-Elmer 9600 thermal cycler with the following cycle: 94° (10 sec), 55° (15 sec), 72° (30 sec). The number of cycles ($PMCA_1$ = 23, and 24-hydroxylase = 25) was determined empirically from preliminary efficiency tests.

Electrophoresis was conducted using 8 μL of PCR products from each reaction that was loaded onto a 5% polyacrylamide gel. The gel was electrophoresed in TBE buffer [53.4 mM Tris–borate and 1.2 mM EDTA (pH 8.0)] for 1 hr at 35 W. Following electrophoresis, the gel was fixed onto Whatman chromatographic paper and dried. Radioactive bands were quantitated with a Packard imager. The log of the radioactive count ratio between target gene product and standard DNA product was plotted against the log of mimic concentration. The equation from linear regression of each curve was used for calculating the RNA concentration in each sample.

Competitive Protein Binding Analysis

Competitive binding assays using the calf thymus 1,25-$(OH)_2D$-receptor and the rat plasma vitamin D binding protein were performed as previously described [13].

EMSAs

Expression of rhVDR and rhRXRα and their use in the shift assay have been described previously [14, 15]. Briefly, the standard binding buffer consisted of 120 mM KCl, 20 mM Tris (pH 7.5), 1.5 mM EDTA, 2 mM DTT, 5% glycerol, 0.5% CHAPS, 10 mM NaF, 100 μM Na_3VO_4, 0.5 mg dIdC, 0.5 mM leupeptin, and 250 nM vitamin D compound in a 20-μL volume. All samples were incubated at 4°. Cytosols of recombinant receptor extracts were diluted 1:50 into a KTEDG buffer solution [400 mM KCl, 20 mM Tris (pH 7.5), 1 mM EDTA, 2 mM DTT, and 10% glycerol] prior to use. Aliquots of the diluted receptor mixture (2 μL per 20-mL binding sample) were mixed with the appropriate vitamin D-containing solution to yield the standard binding buffer indicated above. Following 30 min in this buffer, the radiolabeled 24-hydroxylase probe was added, and the incubation continued for an additional 30 min. Then the samples were applied to cooled, pre-run 5% polyacrylamide gels (29:1) in 0.5x TBE buffer, and electrophoresis was initiated at 14 V/cm for 2.5 hr. Gels were transferred and dried, and autoradiography was performed. The radiolabeled rat 24-hydroxylase VDRE probe was prepared by annealing synthetic oligonucleotides possessing 4-bp overhangs: top strand, 5′-TCG AGC GGC GCC CTC ACT CAC CTC GCG-3′. End-labeling was

TABLE 1. Plasma calcium and vitamin D sterol concentrations in rats dosed with 1 ng/g body weight of either 1,24-$(OH)_2D_3$, 1,25-$(OH)_2D_3$, or 1,24-$(OH)_2D_2$

Treatment	Calcium* (mg/dL)	1,24-$(OH)_2D_2$*	1,24-$(OH)_2D_3$	1,25-$(OH)_2D_3$*	Total 1α-hydroxylated*
		(pg/mL)			
Control	10.7 ± 0.1[a]	8 ± 4[a]	<4	116 ± 10[a]	128 ± 12[a]
1,24-$(OH)_2D_2$	10.6 ± 0.2[a]	43 ± 10[b]	<4	52 ± 22[b]	99 ± 20[a]
1,24-$(OH)_2D_3$	11.1 ± 0.3[b]	7 ± 7[a]	88 ± 22	23 ± 8[b]	118 ± 35[a]
1,25-$(OH)_2D_3$	11.4 ± 0.3[b]	<4	<4	377 ± 61[c]	385 ± 62[b]

Samples were obtained 4–5 hr following the last of the seven daily doses. Values are means ± SEM, N = 5.
*Values in columns with different superscripts are significantly different, $P \leq 0.05$.

achieved by the fill-in reaction using [^{32}P]-dATP in combination with Klenow fragment. Annealed, radiolabeled probes were then gel-purified prior to their use. The assays were repeated four times for each compound.

Statistics

The effect of treatment was analyzed by ANOVA, and transformed treatment means were compared using Statview 4.5 (SAS Institute).

RESULTS

Vitamin D Metabolites

Table 1 summarizes the concentrations of the 1α-hydroxylated metabolites measured 4–5 hr following the last of the seven daily doses. Changes in plasma vitamin D metabolites reflected the treatments imposed on the different groups (Table 1). Plasma 1,24-$(OH)_2D_2$ was elevated from a concentration of 8 pg/mL (controls) to 43 pg/mL in 1,24-$(OH)_2D_2$-treated rats. Likewise, 1,24-$(OH)_2D_3$ was elevated from < 4 to 88 pg/mL in 1,24-$(OH)_2D_3$-treated animals. Plasma concentrations of 1,25-$(OH)_2D_3$ were elevated from 116 to 377 pg/mL in 1,25-$(OH)_2D_3$-treated rats. Plasma 1,25-$(OH)_2D_3$ was also depressed significantly ($P < 0.05$) relative to controls, in rats treated with 1,24-$(OH)_2D_2$ to 52 pg/mL and in rats treated with 1,24-$(OH)_2D_3$ to 23 pg/mL. The depression in plasma 1,25-$(OH)_2D_3$ appeared to be more pronounced in the 1,24-$(OH)_2D_3$-treated group, although not significantly. Total 1α-hydroxylated metabolites are also summarized in Table 1. These numbers reflect the combined concentrations of the three metabolites measured. As shown, relative to controls, the total 1α-hydroxylated metabolites remained relatively unchanged in the 1,24-$(OH)_2D_2$- and 1,24-$(OH)_2D_3$-treated rats, but were elevated in the 1,25-$(OH)_2D_3$-treated rats.

Calcium

Plasma calcium was elevated in the rats treated with 1,24-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$, but not in the 1,24-$(OH)_2D_2$-dosed rats. Urinary calcium (expressed as total calcium excreted per day or micrograms calcium per milligram of creatinine) was elevated significantly (relative to controls) in all groups (Fig. 3). The order of activity was 1,25-$(OH)_2D_3 \geq$ 1,24-$(OH)_2D_3 >$ 1,24-$(OH)_2D_2$.

mRNA

The mRNA was quantitated for 24-hydroxylase and $PMCA_1$ in intestinal and kidney tissues (Fig. 4). Duodenal 24-hydroxylase mRNA was elevated in all groups relative to controls; however, the elevations were significantly higher in the 1,24-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ groups compared with the 1,24-$(OH)_2D_2$ group. Kidney 24-hydroxylase also was elevated significantly in the 1,24-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ groups relative to the control and the 1,24-$(OH)_2D_2$ groups. Duodenal $PMCA_1$ was elevated in all groups relative to controls. There were,

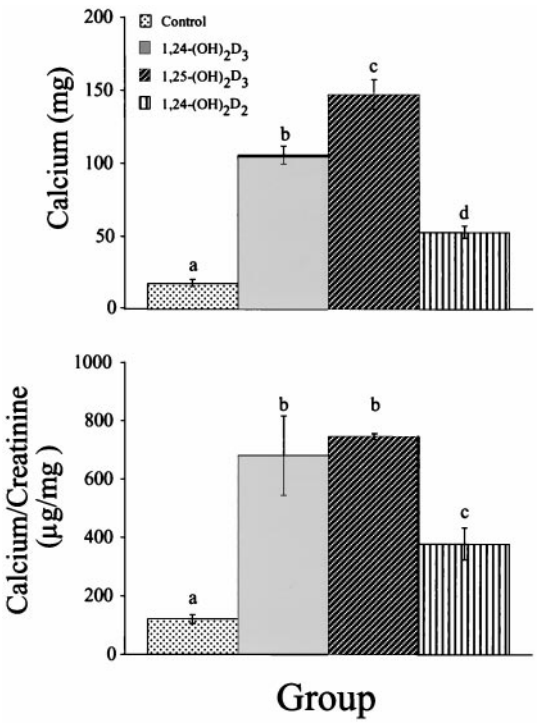

FIG. 3. Cumulative calcium excretion (days 3–7 of treatment) expressed as milligrams per day (upper panel) or micrograms per milligram of creatinine (lower panel) in control rats and those treated with 1,24-$(OH)_2D_2$, 1,24-$(OH)_2D_3$, or 1,25-$(OH)_2D_3$. Data represent the means ± SEM of 6 rats/group. Values not sharing a common letter are significantly different ($P \leq 0.05$).

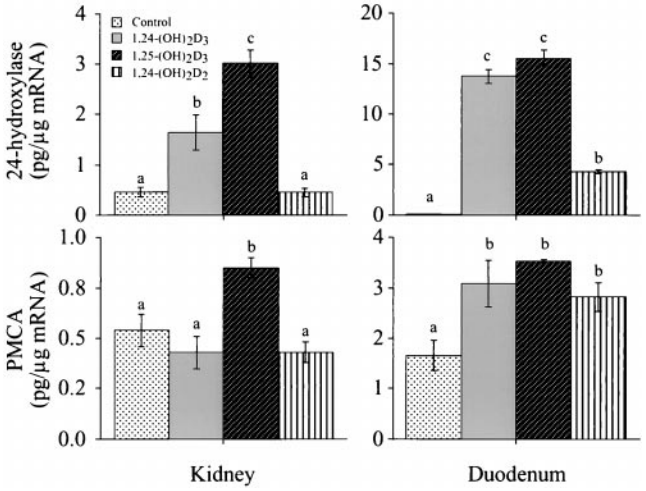

FIG. 4. Kidney and duodenal 24-hydroxylase and $PMCA_1$ mRNA concentrations in control rats and those treated with 1,24-$(OH)_2D_2$, 1,24-$(OH)_2D_3$, or 1,25-$(OH)_2D_3$. Data represent the means ± SEM of 3 rats/group. Values not sharing a common letter are significantly different ($P \leq 0.05$).

however, no significant differences between groups in this parameter. In contrast, kidney $PMCA_1$ was elevated in only the 1,25-$(OH)_2D_3$ group.

Competitive Protein Binding Assay

The relative ability of 1,24-$(OH)_2D_3$ and 1,24-$(OH)_2D_2$ to compete for binding sites on the vitamin D receptor prepared from calf thymus was examined. As shown in Fig. 5A, 1,24-$(OH)_2D_3$ had ~2-fold higher affinity for binding to the receptor than 1,25-$(OH)_2D_3$ and ~2.5 fold greater affinity than 1,24-$(OH)_2D_2$. We also examined the ability of the metabolites to compete for binding to the plasma vitamin D binding protein (Fig. 5B). In this assay, 1,25-$(OH)_2D_3$ had ~10% greater affinity for binding sites than either 1,24-$(OH)_2D_2$ or 1,24-$(OH)_2D_3$. The latter two metabolites had equal affinity for the plasma vitamin D binding protein.

EMSA Analysis of Metabolite/rhVDR/rhRXR Complex

Binding of either 1,25-$(OH)_2D_3$ or 1,24-$(OH)_2D_3$ to the rhVDR/rhRXR heterodimer stimulated ($P \leq 0.05$) the mobility of the 24-hydroxylase VDRE/heterodimer complex when compared with the ligand-free complex (Table 2, Fig. 6A). Binding by 1,24-$(OH)_2D_2$ also increased the mobility of the complex, but not significantly. These observed ligand-induced mobility changes were not related to gel "smiling" and were independent of the order of loading or position on the gel. The graph (Fig. 6B) represents the densitometric scans of the individual lanes. Intensities of the bound bands were approximately equal for 1,25-$(OH)_2D_3$ and 1,24-$(OH)_2D_2$, both of which were approximately 20% higher than the band intensity exhibited in the absence of ligand. 1,24-$(OH)_2D_3$, however, had

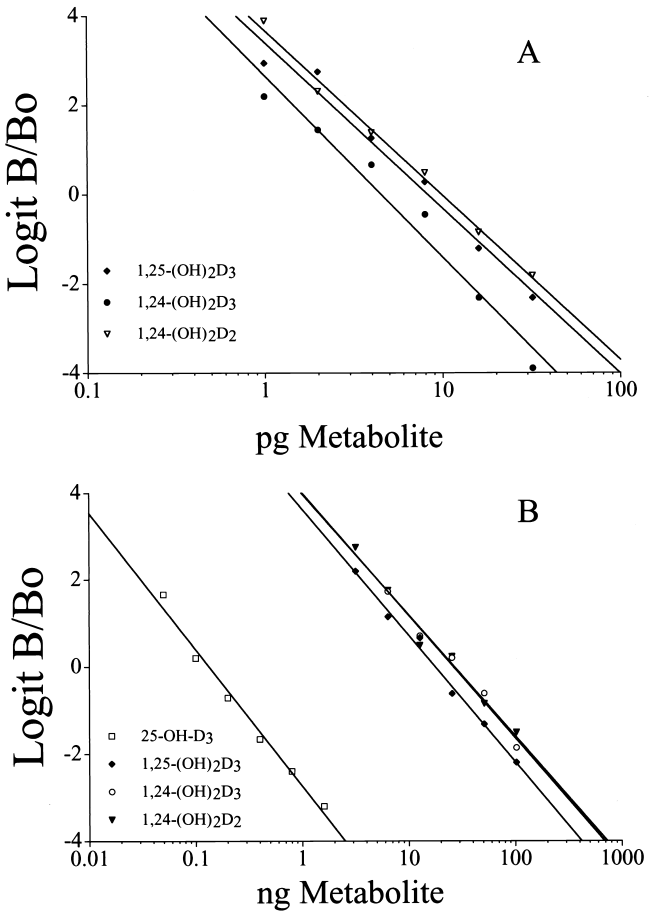

FIG. 5. Displacement potency of 1,24-$(OH)_2D_2$, 1,24-$(OH)_2D_3$, and 1,25-$(OH)_2D_3$ using (A) the calf thymus as a source of the 1,25-dihydroxyvitamin D receptor, and (B) rat plasma as a source of the plasma vitamin D binding protein.

a higher band intensity than either 1,25-$(OH)_2D_3$ or 1,24-$(OH)_2D_2$. Statistical evaluation of the maximum intensities for each treatment is summarized in Table 2. Although the intensities were higher for 1,25-$(OH)_2D_3$ and 1,24-$(OH)_2D_2$, they were not statistically different ($P > 0.05$) from the control. The band intensity resulting from the 1,24-$(OH)_2D_3$ treatment, however, was different from controls ($P \leq 0.05$).

TABLE 2. Effect of 1,25-$(OH)_2D_3$, 1,24-$(OH)_2D_3$, and 1,24-$(OH)_2D_2$ on EMSA mobility of rhVDR/rhRXR heterodimers bound to 24-hydroxylase VDRE

Treatment	Mobility* (pixels)	Maximum intensity* (arbitrary units)
Control	197 ± 0.9[a]	144 ± 13[a]
1,25-$(OH)_2D_3$	208 ± 1.2[b]	168 ± 5.5[a]
1,24-$(OH)_2D_3$	206 ± 3.0[b]	182 ± 7.2[b]
1,24-$(OH)_2D_2$	203 ± 2.5[a]	168 ± 2.6[a]

Data are presented as means ± SEM, N = 4.
*Values in columns with different superscripts are significantly different, $P \leq 0.05$.

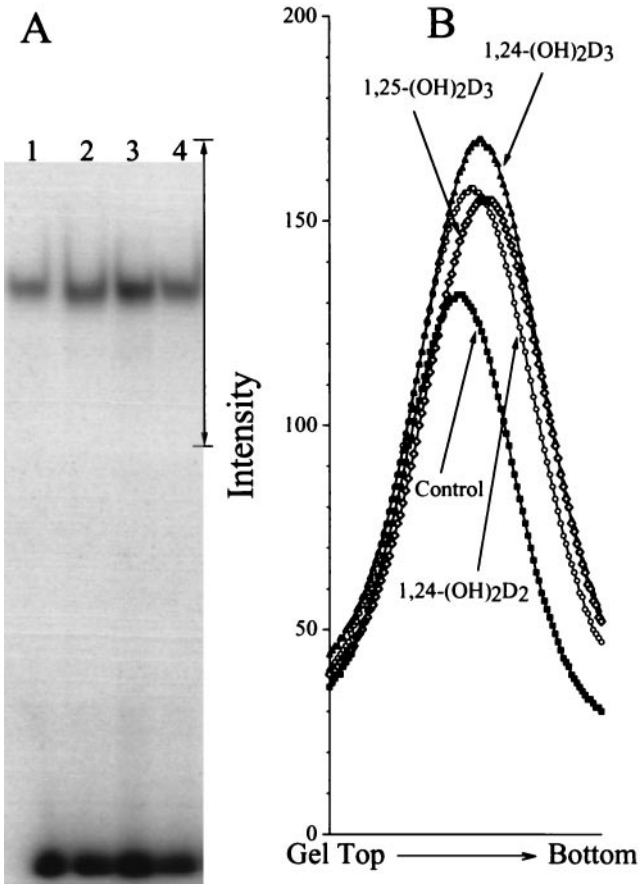

FIG. 6. EMSA analysis of metabolite bound to rhVDR/rhRXR heterodimer. (A) The heterodimer complex was bound to the 24-hydroxylase VDRE in the absence of metabolite (control, lane 1) or in the presence of 250 nM 1,25-$(OH)_2D_3$ (lane 2), 1,24-$(OH)_2D_3$ (lane 3), and 1,24-$(OH)_2D_2$ (lane 4). (B) Autoradiograph from (A) was scanned over the entire length, but only the region encompassing the bound probe is presented to highlight the mobility differences.

DISCUSSION

1,24-$(OH)_2D_2$ and 1,24-$(OH)_2D_3$ are vitamin D metabolites currently being investigated for use in treating a variety of human diseases that are responsive to vitamin D [6–9, 16–18]. Successful use of these and other vitamin D-related compounds relies not only on their ability to cure the diseases of interest but also in avoiding unwanted and potentially dangerous side-effects such as hypercalcemia. To that end, we tested the ability of 1,24-$(OH)_2D_2$ and 1,24-$(OH)_2D_3$ to effect changes in several biological parameters related to calcium and vitamin D metabolism. Previous studies comparing 1,24-$(OH)_2D_2$ or 1,24-$(OH)_2D_3$ to 1,25-$(OH)_2D_3$ in vitamin D-deficient and/or vitamin D-replete rats found that the 24-hydroxylated analogs produced smaller increases in calcium levels than did 1,25-$(OH)_2D_3$ [3, 5–9]. The present study compared 1,24-$(OH)_2D_2$ and 1,24-$(OH)_2D_3$ with each other as well as with 1,25-$(OH)_2D_3$ in vitamin D-replete rats. The results indicated that 1,24-$(OH)_2D_2$ is less potent than either 1,24-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ in producing hypercalcemia or hypercalciuria in young growing rats, and that 1,24-$(OH)_2D_3$ was similar to or slightly less active than 1,25-$(OH)_2D_3$ in this respect (Table 1, Fig. 3). Surprisingly, the degree of hypercalcemia and hypercalciuria induced by these compounds was not related to plasma concentrations of the vitamin D sterols with which the animals were being treated. For example, 1,25-$(OH)_2D_3$ and 1,24-$(OH)_2D_3$ were similar in their ability to increase plasma and urinary calcium; however, their effect on plasma levels of 1α-hydroxylated compounds differed. Plasma concentration of 1,25-$(OH)_2D_3$, and, therefore, total 1α-hydroxylated compounds, was elevated 3-fold from 116 pg/mL in controls to a peak of 377 pg/mL in rats treated with 1,25-$(OH)_2D_3$, whereas the plasma level of total 1α-hydroxylated compounds was unchanged in 1,24-$(OH)_2D_3$-treated rats (Table 1). 1,24-$(OH)_2D_2$, which had the least effect on plasma and urinary calcium perturbations, also did not change the levels of total 1α-hydroxylated compounds. The reasons for the differences in peak analog concentrations achieved with each treatment are unknown. Several factors could be involved, including absorption, tissue sequestration, metabolism, and/or binding to the plasma vitamin D binding protein. There is little or no information regarding absorption and tissue sequestration of 1-hydroxylated vitamin D analogs, primarily due to the lack of appropriate radiolabeled material. One preliminary report, however, did indicate that 1α-OH-D_2 and 1α-OH-D_3 had similar bioavailability when given orally [19]. There is, however, information regarding further metabolism of these compounds. For example, it has been demonstrated that 1,25-$(OH)_2D_3$ and 1,24-$(OH)_2D_3$ are both metabolized to 1,24,25-$(OH)_3D_3$ [20, 21], while 1,24-$(OH)_2D_2$ is metabolized preferentially to 1,24,26-$(OH)_3D_2$. The 25-hydroxylation of 1,24-$(OH)_2D_2$ to form 1,24,25-$(OH)_3D_2$ appears to be minimal or nonexistent [22]. The 1,24,25-$(OH)_3D_3$ has biological activity [20], whereas 1,24,25$(OH)_3D_2$ and 1,24,26-$(OH)_3D_2$ are relatively inert [22, 23]. These differences in metabolic pathways and the speed at which they occur may partially explain the differences in plasma concentrations as well as their calcemic activity. In addition, both 1,24-$(OH)_2D_2$ and 1,24-$(OH)_2D_3$ have lower affinity for the plasma vitamin D binding protein than 1,25-$(OH)_2D_3$ (Fig. 5B). Lower affinity for the plasma vitamin D binding protein is known to render vitamin D analogs more readily available for target tissue distribution and metabolism [24]. These characteristics could also help explain the dissociation between plasma concentration and biological response.

The levels of vitamin D-24-hydroxylase were also affected differently by 1,24-$(OH)_2D_2$ relative to the other analogs. 1,24-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ produced similar increases in duodenal and kidney 24-hydroxylase mRNA, and these increases were significantly greater than the response produced by 1,24-$(OH)_2D_2$. In contrast, changes in the levels of enzymes involved in calcium and vitamin D metabolism, namely the 25-hydroxyvitamin D-1α-hydroxylase and PMCA, were similar for 1,24-

$(OH)_2D_2$ and 1,24-$(OH)_2D_3$. The reduction in the endogenous levels of 1,25-$(OH)_2D_3$ by 1,24-$(OH)_2D_2$ and 1,24-$(OH)_2D_3$ (Table 1) may be viewed as a measure of a systemic effect, namely, the capacity of the analog to inhibit the renal 25-hydroxyvitamin D-1α-hydroxylase. As shown in Table 1, the plasma level of 1,25-$(OH)_2D_3$ was lowered by both of these compounds. While 1,24-$(OH)_2D_3$ appeared to have a greater effect, the reduction produced by 1,24-$(OH)_2D_3$ and 1,24-$(OH)_2D_2$ was not statistically different. Furthermore, the mRNA for PMCA in the duodenum was up-regulated significantly to about the same level by all sterols. PMCA is thought to play an integral role in the transport of calcium across the intestine [20]; however, most of the research on this enzyme has used the vitamin D-deficient rat. While 1,25-$(OH)_2D_3$ increased the mRNA for this enzyme in the kidney as well, neither of the 24-hydroxylated analogs elicited this response in the vitamin D-replete rats. Nonetheless, the data from the duodenum suggested that 1,24-$(OH)_2D_2$, as well as 1,24-$(OH)_2D_3$, can up-regulate some vitamin D-responsive genes to the same degree as 1,25-$(OH)_2D_3$.

It appears, therefore, that 1,24-$(OH)_2D_2$ and 1,24-$(OH)_2D_3$ can produce differing effects on serum and urine calcium and on the induction of mRNA for the vitamin D-24-hydroxylase, but similar effects on the activity of 25-hydroxyvitamin D-1α-hydroxylase and induction of mRNA for PMCA. The mechanism(s) by which these and other vitamin D analogs produce differing biological effects is the subject of much current investigation. The efficiency of 1,24-$(OH)_2D_3$ at eliciting changes in plasma and urinary calcium was quite dramatic, particularly when considering the relatively minor changes in plasma concentrations seen in rats treated with this metabolite. Inspection of the data in Table 2 and Figs. 2 and 6 may provide some insights as to the possible mechanisms of the apparent activity of 1,24-$(OH)_2D_3$. Two molecular events that are important to the biological action of vitamin D metabolites are binding to VDR and binding of the VDR/RXR complex to DNA. The VDR binding data in Fig. 2 suggest that 1,24-$(OH)_2D_3$ has ~2-fold greater affinity than 1,25-$(OH)_2D_3$ for calf thymus VDR. This result was similar to those reported earlier by Matsumoto *et al.* [7], who used receptor isolated from human keratinocytes, and by Binderup [25], who used receptor isolated from rachitic chicken. In addition to enhanced receptor affinity, binding of the rhVDR/rhRXR complex to the 24-hydroxylase VDRE was enhanced significantly by 1,24-$(OH)_2D_3$ relative to controls, as demonstrated by the increased band intensity observed in the EMSA analysis (Table 2, Fig. 6). In this assay, both 1,24-$(OH)_2D_2$ and 1,25-$(OH)_2D_3$ produced similar maximum intensities and were elevated but were not statistically different relative to controls. Therefore, 1,24-$(OH)_2D_3$ may evoke a more potent biological response as a result of enhanced VDR binding and increased avidity of binding between the VDR/RXR complex and the VDRE.

In conclusion, these data indicate that 1,24-$(OH)_2D_2$ is less hypercalcemic and less hypercalciuric, and its ability to up-regulate the tissue 24-hydroxylase was also attenuated relative to 1,24-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$. The ability of 1,24-$(OH)_2D_2$ to up-regulate the intestinal CaATPase was, however, indistinguishable from that of either 1,24-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$. Treatment with either 1,24-$(OH)_2D_2$ or 1,24-$(OH)_2D_3$ produced a similar reduction in endogenous 1,25-$(OH)_2D_3$ plasma levels. These data suggest that 1,24-$(OH)_2D_2$ produces smaller increases in calcium levels in young growing rats; however, 1,24-$(OH)_2D_2$ can evoke other biological responses similar to the vitamin D_3 sterols. 1,24-$(OH)_2D_2$ also induced conformational changes in the VDR/RXR complex that were different from those induced by either 1,24-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$. These different conformational states may explain why some vitamin D metabolites and/or analogs can up-regulate some vitamin D-responsive genes but at the same time be less hypercalcemic.

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