Vol. 132, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1985 Pages 198-203

EVALUATION OF A PHOTOLABILE DERIVATIVE OF 1,25-DIHYDROXYVITAMIN D_3 AS A PHOTOAFFINITY PROBE FOR 1,25-DIHYDROXYVITAMIN-D_3 RECEPTOR IN CHICK INTESTINAL CYTOSOL

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Received August 3, 1985

Summary: We evaluated the viability of 1α,25-dihydroxyvitamin D_3-3β-[N-(4-azido-2-nitrophenyl)glycinate] (1,25-$(OH)_2$-D_3-ANG), an analog of 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$-D_3) as a photoaffinity probe for 1,25-$(OH)_2$-D_3 receptor in chick intestinal cytosol. A competitive-binding assay revealed that chick intestinal cytosolic 1,25-$(OH)_2$- D_3 receptor bound to 1,25-$(OH)_2$-D_3-ANG approximately 20-times less effectively than it did to 1,25-$(OH)_2$-D_3. Irradiation of 1,25-$(OH)_2$-D_3- ANG in the presence of chick intestinal cytosolic preparation significantly diminished subsequent binding to ^{3}H-1,25-$(OH)_2$-D_3, suggesting that the photoaffinity analog was covalently attached to the receptor. Therefore the nitroarylazide derivative of 1,25-$(OH)_2$-D_3 may be a valuable photoaffinity probe for the characterization of the 1,25-$(OH)_2$-D_3 receptor.

Introduction: It is established that vitamin D_3 undergoes sequential metabolism to 25-hydroxyvitamin D_3 (25-OH-D_3) in the liver and then to 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$-D_3) in the kidney (1,2). A receptor-like protein having high affinity and low capacity for 1,25-$(OH)_2$-D_3 was identified in the intestinal cytosol and the nuclei (1,2).

Photoaffinity labelling of steroid and peptide hormone receptors has been extremely valuable in localizing and isolating membrane-bound, nuclear and cytoplasmic receptors and for structure--function analyses (3). We initiated a program to synthesize a photoaffinity-labelled derivative of 1,25-$(OH)_2$-D_3. Based on previous structure--activity analyses for the chick intestinal 1,25-$(OH)_2$-D_3 receptor (2) that showed that the 1α-OH and 25-OH are important recognition markers, we derivatized the 3β-OH via an ester linkage with a glycine analog containing a 4-azido-2-nitro-phenyl moiety. We report

0006-291X/85 $1.50

that 1,25-dihydroxyvitamin D_3-3β-[N-(4-azido-2-nitrophenyl)]glycinate ($1,25\text{-}(OH)_2\text{-}D_3$-ANG) is recognized by the $1,25\text{-}(OH)_2\text{-}D_3$ receptor, and is effective in diminishing the binding activity of the $1,25\text{-}(OH)_2\text{-}D_3$ receptor when it is photolyzed with the receptor.

MATERIALS AND METHODS: 1α,25-dihydroxyvitamin D_3 was a generous gift from Dr. Milan Uskokovic, Hoffmann-La Roche Inc., Nutley, NJ. $1,25\text{-}(OH)_2\text{-}[26,27\text{-}^3H]\text{-}D_3$ (specific activity 158--170 Ci/mmol) was purchased from New England Nuclear, Boston, MA. Photolysis of the samples was performed in quartz tubes in a Rayonet minireactor (Southern New England Ultraviolet Co., Conn.) unless mentioned otherwise. All the compounds containing light-sensitive azido-nitrophenyl group were handled in vials wrapped in aluminum foil. Cytosolic extract was prepared from chick intestines as described elsewhere (4). Hypertonic buffer was prepared with 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM dithiothreotol, 300 mM KCl, and 10 mM sodium molybdate. Lambert's charcoal solution was made from 5% neutralized Norit decolorizing charcoal 0.5% Dextran T-70, 25% normal human plasma, in 0.05 M phosphate buffer containing 0.1 M KCl, pH 7.4.

Chemical synthesis and characterization of $1,25\text{-}(OH)_2\text{-}D_3$-ANG was reported previously (5).

Competitive-binding assays for $1,25\text{-}(OH)_2\text{-}D_3$ and $1,25\text{-}(OH)_2\text{-}D_3$-ANG. The assays were performed with a chick intestinal cytosolic preparation (diluted 1:40 with hypertonic buffer), $^3H\text{-}1,25\text{-}(OH)_2\text{-}D_3$ (8400 cpm, sp. ac. 158 Ci/mmol), with various concentrations of $1,25\text{-}(OH)_2\text{-}D_3$ (5 fmol -- 24 pmol) or $1,25\text{-}(OH)_2\text{-}D_3$-ANG (80 pmol--158 pmol) according to previously published procedures (6).

Evaluation of the efficiency of charcoal treatment to remove $1,25\text{-}(OH)_2\text{-}D_3$ ANG from the receptor site and to regenerate the receptor for further binding to $^3H\text{-}1,25\text{-}(OH)_2\text{-}D_3$. 200 µl of cytosol (diluted 1:10 with hypertonic buffer) was placed in each of three tubes. The first sample (control) was incubated with $^3H\text{-}1,25\text{-}(OH)_2\text{-}D_3$ (10,000 cpm, sp. ac. 158 Ci/mmol in 20 µl of ethanol) for 60 min at 25°C followed by cooling on ice, treatment with ice-cold Lambert charcoal (75 µl, 20 min at 0°C), centrifugation (3000 x g, 4°C, 10 min), and counting of the supernatant for radioactivity. The second and third samples were preincubated with $1,25\text{-}(OH)_2\text{-}D_3$-ANG (20 pmole) or $1,25\text{-}(OH)_2\text{-}D_3$ (0.6 pmole) for 60 min at 25°C followed by incubation with Lambert's charcoal to remove unbound sterols. The supernatants were incubated with $^3H\text{-}1,25\text{-}(OH)_2\text{-}D_3$ and analyzed as in the case of the control.

Assay for receptor blockade by irradiated $1,25\text{-}(OH)_2\text{-}D_3$-ANG. $1,25\text{-}(OH)_2\text{-}D_3$-ANG (5 pmol/20 µl ethanol) was added to each of eight quartz tubes containing one ml of chick intestinal cytosol (diluted 1:90 with hypertonic buffer). The solutions were incubated at 25°C for 30 min and then four tubes were irradiated for 15 min. Then all the tubes were briefly cooled on ice treated with Lambert charcoal (300 µl, 0°C, 30 min) and centrifuged (3000 x g, 4°C, 10 min). One ml of supernatant was withdrawn from each tube and mixed with $^3H\text{-}1,25\text{-}(OH)_2\text{-}D_3$ (200,000 cpm/25 µl of ethanol, sp. ac. 158 Ci/mmol). The tubes were incubated for 60 min at 25°C, cooled briefly on ice, treated with ice-cold Lambert's charcoal (30 min, 0°C), centrifuged (3000 x g, 4°C, 10 min), and 150 µl of supernatant from each tube was counted for radioactivity.

Sucrose-Density-Gradient-Sedimentation Analysis. Two quartz tubes each containing 400 µl of chick intestinal cytosol and $1,25\text{-}(OH)_2\text{-}D_3$-ANG (158 pmol in 20 µl ethanol) were incubated at 25°C for 60 min. One tube was chilled

on ice and the other was irradiated for 30 min. The cytosols were then treated with ice-cold Lambert charcoal (150 µl, 0°C, 15 min), centrifuged (3000 x g, 4°C, 10 min), and 400 µl supernants were incubated with ^{3}H-1,25-$(OH)_2$-D_3 (100,000 cmp/20 µl ethanol, sp. ac. 170 Ci/mmol) at 25°C for 90 min. After the incubation unbound sterol was removed by charcoal treatment as above, and supernatant (150 µl) from each tube was loaded onto 4.8-ml sucrose density gradients (4--20% sucrose in hypertonic buffer). The gradients were centrifuged at 49,000 rpm (257,000 x g) at 4°C for 19 h. After centrifugation, fractions were collected from the bottom of the gradients and counted for radioactivity. Control experiments were done in a similar fashion with two samples, each containing 400 µl of chick intestinal cytosol and ^{3}H-1,25-$(OH)_2$-D_3 (100,000 cpm/20 µl ethanol, sp. ac. 170 Ci/mmol). After initial incubation (25°C, 60 min.), one sample was incubated with cold 1,25-$(OH)_2$-D_3 (24 nmol/20 µl ethanol).

To determine the effect of UV-irradiation on the binding capacity of 1,25-$(OH)_2$-D_3 receptor, a sample of chick intestinal cytosol (400 µl) was irradiated for 30 min followed by incubation with ^{3}H-1,25-$(OH)_2$-D_3 (100,000 cpm/20 µl ethanol, sp. ac. 170 Ci/mmol) and sucrose-density-gradient sedimentation analysis as described above.

RESULTS AND DISCUSSION: The competitive-binding assay using the chick intestinal cytosolic 1,25-$(OH)_2$-D_3 receptor displayed in Fig. 1 shows that approximately 1.9 pmol of 1,25-$(OH)_2$-D_3-ANG was capable of displacing 50% of the maximally bound ^{3}H-1,25-$(OH)_2$-D_3 from the receptor site. The concentration of 1,25-$(OH)_2$-D_3-ANG required to achieve a 50% inhibition of binding of ^{3}H-1,25-$(OH)_2$-D_3 was thus approximately 20-fold greater than that required for 1,25-$(OH)_2$-D_3. Since 1,25-$(OH)_2$-D_3-ANG was capable of

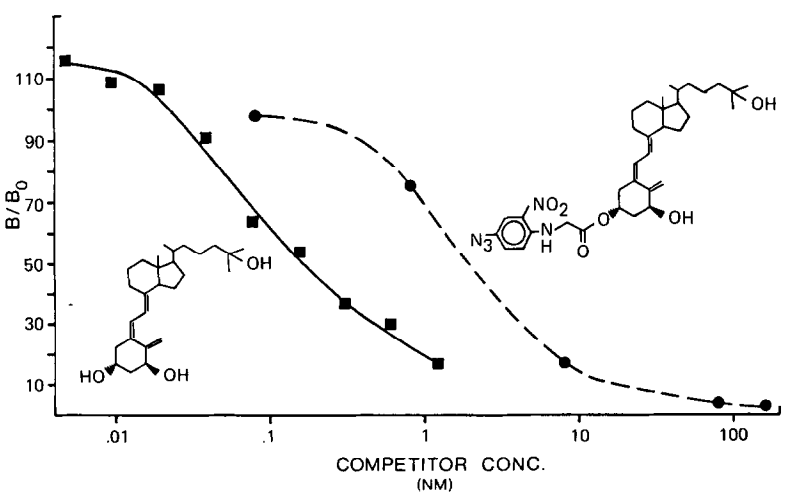

Figure 1: Competitive binding affinity assay for the 1,25-$(OH)_2$-D_3 receptor. Various concentrations of 1,25-$(OH)_2$-D_3 (—■—)and 1,25-$(OH)_2$-D_3-ANG (--●--) were incubated with ^{3}H-1,25-$(OH)_2$-D_3 in chick intestinal cytosol 1 ml, 1:40 in hypertonic buffer). Dextran-coated charcoal was used to absorb unbound materials.

competing with ^{3}H-1,25-$(OH)_2$-D_3 for binding to the chick intestinal cytosol receptor, we were interested in determining whether photoactivation of the 1,25-$(OH)_2$-D_3-ANG in the presence of the 1,25-$(OH)_2$-D_3 receptor caused covalent bonding to the receptor in a manner that would permanently interfere with subsequent binding to the natural ligand. Before we could test this hypothesis we had to first be certain that we could strip, by charcoal treatment, the non-covalently bound 1,25-$(OH)_2$-D_3-ANG from 1,25-$(OH)_2$-D_3 receptor. Incubation of duplicate samples of chick cytosol with 20 pmol of 1,25-$(OH)_2$-D_3-ANG or 0.6 pmole of 1,25-$(OH)_2$-D_3 followed by incubation with Lambert's charcoal to remove unbound secosterols regenerated the capacity of the 1,25-$(OH)_2$-D_3 receptor to bind ^{3}H-1,25-$(OH)_2$-D_3 by 90.5% and 81.5%, respectively. Incubation of the cytosolic receptor with cytosolic receptor with 1,25-$(OH)_2$-D_3-ANG followed by irradiation and charcoal-stripping revealed that only 70$\pm$5% of the capacity of the cytosol to bind ^{3}H-1,25-$(OH)_2$-D_3 was regenerated when compared with similar cytosolic preparations that were not irradiated (4950$\pm$341 cpm bound to the irradiated cytosol vs 7663$\pm$810 cpm to the non-irradiated cytosol; $p<0.03$).

These observations were confirmed by sucrose-density analysis of similarly treated cytosol preparations. As can be seen in Fig. 2A, the intestinal cytosol contained a low capacity 3.7S macromolecular binder for ^{3}H-1,25-$(OH)_2$-D_3, and, as shown in Fig. 2B, exposure of the cytosolic receptor to UV-radiation did not affect its binding to ^{3}H-1,25-$(OH)_2$-D_3. However, when the cytosolic preparation was preincubated with 158 pmole of 1,25-$(OH)_2$-D_3-ANG followed by irradiation before incubation with ^{3}H-1,25-$(OH)_2$-D_3, the amount of ^{3}H-1,25-$(OH)_2$-D_3 that was bound to the 3.7S receptor was diminished by 57% (Fig. 2C) when compared to the cytosolic preparation that was preincubated with 158 pmole of 1,25-$(OH)_2$-D_3-ANG and not exposed to ultraviolet radiation.

Photoaffinity labelling has been used to investigate steroid and peptide hormone receptors, but has not been used for the 1,25-$(OH)_2$-D_3 receptor. 1,25-$(OH)_2$-D_3 has three hydroxyl functions that require selective protection and deprotection to specifically derivatize only one of them. We derivatized

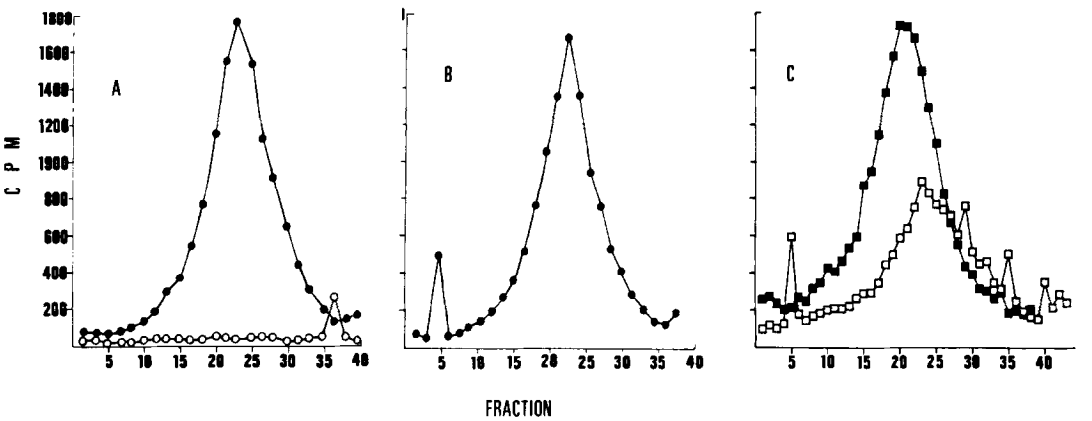

Figure 2: Sucrose density gradient sedimentation profile with chick intestinal cytosol and ^{3}H-1,25-(OH)$_2$-D$_3$ as described in the text. (A) Samples of chick intestinal cytosol were incubated with ^{3}H-1,25-(OH)$_2$-D$_3$ alone (—●—), or in the presence of an excess of 1,25-(OH)$_2$-D$_3$ (—○—). Following treatment with dextran-coated charcoal to remove unbound sterol, aliquots of each sample were run through 4 - 20 % sucrose density gradients. The gradients were then fractionated from the bottom of the tubes. (B) A sample of chick intestinal cytosol was irradiated for 20 mins prior to incubation with (—●—) ^{3}H-1,25-(OH)$_2$-D$_3$. Unbound sterol was removed by charcoal treatment. The supernatant was run through 4-20% sucrose-density gradient and fractionated as in (A). (C) Samples of chick intestinal cytosol containing 1,25-(OH)$_2$-D$_3$-ANG were either irradiated (—□—), or protected from exposure to light (—■—) were treated with charcoal to remove non-covalently bound 1,25-(OH)$_2$-D$_3$-ANG prior to incubation with ^{3}H-1,25-(OH)$_2$-D$_3$. After the incubation, the samples were treated with dextran-coated charcoal, run through 4 - 20 % sucrose-density gradients and fractionated by procedures as in (A).

the hormone at the 3β-OH position because previous structure--activity studies with the 1,25-(OH)$_2$-D$_3$ receptor had revealed that the 1α-OH and 25-OH were most important as recognition sites for the receptor to bind to the substrate (2). Nitroarylazide was chosen as the photolabile moiety because (a) its stability in the dark in solution was excellent, (b) its susceptibility to intramolecular rearrangement after photolysis was very low, and (c) the wavelength for photolysis was greater than 320 nm, which made it unlikely that the 5,6-cis-triene system would also be photolyzed during the coupling reaction. When designing this molecule, there was concern that the bulky 4-azido-2-nitrophenyl glycinate group at the 3β position would be so large that

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wavelength for photolysis was greater than 320 nm, which made it unlikely that the 5,6-cis-triene system would also be photolyzed during the coupling reaction. When designing this molecule, there was concern that the bulky 4-azido-2-nitrophenyl glycinate group at the 3β position would be so large that it would prevent the 1,25-$(OH)_2$-D_3 receptor from binding to it. However, an analysis of the binding affinity of the chick intestinal cytosolic 1,25-$(OH)_2$-D_3 receptor for the photoaffinity analog revealed that the addition of the bulky nitroarylazide group decreased its recognition by only 20-fold (Fig. 1).

These data suggest that the 1,25-$(OH)_2$-D_3-ANG links covalently to the 1,25-$(OH)_2$-D_3 cytosolic receptor upon irradiation that in turn prevents the further binding of the receptor to ^{3}H-1,25-$(OH)_2$-D_3. This raises the possibility of starting with radiolabelled 1,25-$(OH)_2$-D_3-ANG for the specific labelling of the 1,25-$(OH)_2$-D_3 receptor. We are investigating the potential of this technique for a more accurate identification, molecular characterization, and mapping of this hormone receptor.

ACKNOWLEDGEMENTS:

This work was supported by NIH grants No. 7-R01 AM36963 and No. 7 R01 AM369885.

We wish to express our thanks to Dr. Michael Rosenblatt for his many helpful suggestions regarding the synthesis of the 1,25-$(OH)_2$-D_3 photoaffinity probe.

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