Localization of Receptors for 1,25-Dihydroxyvitamin D₃ along the Rat Nephron

DIRECT EVIDENCE FOR PRESENCE OF THE RECEPTORS IN BOTH PROXIMAL AND DISTAL NEPHRON

(Received for publication, May 28, 1982)

Hiroyuki Kawashima and Kiyoshi Kurokawa

From the Nephrology Section, Medical and Research Services, Veterans Administration Wadsworth Medical Center, Los Angeles, California 90073 and Department of Medicine, University of California School of Medicine, Los Angeles, California 90024

We recently demonstrated that 25-hydroxyvitamin D₃-24-hydroxylase, which can be further induced by 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), is localized exclusively in the proximal nephron of the vitamin D-replete rat kidney. These data and the proposed mode of action of 1,25(OH)₂D₃ predict the presence of a receptor for 1,25(OH)₂D₃ in the proximal nephron. However, autoradiographic studies failed to detect significant nuclear uptake of 1,25(OH)₂D₃[³H]D₃ in the proximal nephron.

To localize and characterize receptors for 1,25(OH)₂D₃ along the nephron, preparations of both microdissected defined nephron segments and isolated tubules from the whole kidney of vitamin D-deficient rats were incubated with 1,25(OH)₂D₃[³H]D₃, and specific uptake of the steroid was assessed in the cytosol preparation of tubular cells sonicated in a hypertonic solution. The uptake of 1,25(OH)₂D₃[³H]D₃ was also examined in three defined single nephron segments: proximal convoluted tubules, medullary thick ascending limb of Henle's loop, and collecting tubules. Both proximal convoluted tubules and medullary thick ascending limb of Henle's loop, and collecting tubules. Both proximal convoluted tubules and medullary thick ascending limb of Henle's loop showed a significant uptake of 1,25(OH)₂D₃[³H]D₃. These data demonstrate the presence of 1,25(OH)₂D₃ receptors in both proximal and distal nephron.

The mechanism of action of 1,25(OH)₂D₃ involves binding of this hormone to cytoplasmic receptors and migration of the steroid-receptor complex to the nucleus leading to the induction of hormone-specific protein synthesis (1-4). Although the intestine and bone have generally been considered to be the major target organs, certain data suggest that 1,25(OH)₂D₃ may also act on the kidney (5, 6). Recent studies demonstrated the presence of receptors for 1,25(OH)₂D₃ in the kidney of mouse (7), rat (8), and chick (9), as well as the nuclear transfer of the 1,25(OH)₂D₃-receptor complex in mouse kidney (10). It is also known that at least two distinct proteins, i.e. 25(OH)D₃-24-hydroxylase and calcium-binding protein, are induced by 1,25(OH)₂D₃ in the kidney (1-3).

Recent autoradiographic data demonstrated that the nuclear uptake of 1,25(OH)₂D₃[³H]D₃ in the kidney is predominantly localized to the distal nephron and to some extent to the glomerulus (11). These data are consistent with findings of the exclusive localization of vitamin D-dependent, calcium-binding protein in the distal nephron by immunohistochemical methods in the kidney of mouse (12), rat (13), and chick (14). Another protein induced by 1,25(OH)₂D₃, 25(OH)D₃-24-hydroxylase, has been exclusively localized to the proximal nephron (15).

Therefore, it seems reasonable that either a receptor for 1,25(OH)₂D₃ or another mechanism to recognize 1,25(OH)₂D₃ must be present in the proximal nephron in order to transfer the hormonal signal to the site of the protein synthesis. The present study was carried out aimed at the localization of receptors for 1,25(OH)₂D₃ along the nephron using defined single nephron segments; this was done to obtain further insight into the specific sites of action of 1,25(OH)₂D₃ in the kidney.

EXPERIMENTAL PROCEDURES

Materials—1,25-Dihydroxy-[26,27-methyl-³H]-vitamin D₃ (160 Ci/mmol) and 25-hydroxy-[26,27-methyl-³H]-vitamin D₃ (160 Ci/mmol) were purchased from Amersham Corp. and New England Nuclear, respectively. 1,25(OH)₂D₃, 25(OH)D₃, and 24,25(OH)₂D₃ were generous gifts of Dr. Anthony W. Norman, University of California, Riverside. Human a-globulin fraction IV was purchased from Miles Laboratories (Elkhart, IN). [³C]Ovalbumin was purchased from New England Nuclear.

Animals—Male weanling Holtzman rats were maintained for 3-5 weeks on a vitamin D-deficient diet containing 0.45% calcium and 0.3% phosphorus (15).

Preparation of Isolated Tubular Suspension—Isolated tubules were prepared according to a modification of previously reported methods (16). The animals were anesthetized with intraperitoneal sodium pentobarbital, 40 mg/kg of body weight. The superior mesenteric artery was ligated, and, immediately before perfusion of the kidneys, the aorta was ligated above the renal arteries. The kidneys were then perfused with 30 ml of chilled KRB buffer, pH 7.4, equilibrated with 95% O₂/5% CO₂. The kidneys were removed, minced, and

13428
incubated in the KRB buffer containing 0.1% collagenase (Sigma) at 37 °C for 45 min with gentle shaking in a 25-ml Erlenmeyer flask. At the end of incubation, the incubation mixture was gently pipetted and filtered by a nylon mesh. The filtrate was centrifuged at 750 × g for 3 min. The pellet was washed in Hanks' solution gently three times, and the final pellet was resuspended in Hanks’ solution containing 8.3 mm glucose. This preparation represents a mixture of isolated fragments of tubules of the whole kidney.

Preparation of Defined Single Nephron Segments—Perfused kidneys were removed and sliced (approximately 0.5-1.0 mm in thickness) according to methods that have been reported in detail elsewhere (15, 17). Slices were incubated, for 30 min at 30 °C to KRB buffer containing 8.3 mm glucose, 0.1% bovine serum albumin, and 0.1% collagenase, with constant bubbling with 95% O2/5% CO2. Kidney slices were then rinsed three times with 30-50 ml of ice-cold Hanks’ solution, and defined single nephron segments were dissected freehand under a stereomicroscope in the ice-cold Hanks’ solution.

Uptake of 1,25(OH)2D3 by Isolated Tubules or by Defined Single Nephron Segments—1,25(OH)2D3 (160 Ci/mmol), with or without 200-fold unlabeled 1,25(OH)2D3, was placed in a 5-ml polyethylene tube and dried under an N2 stream. The amount of labeled 1,25(OH)2D3 per incubation was adjusted depending on the purpose and design of the experiment as indicated under “Results.” A 0.5-ml aliquot of tubule suspension of the whole kidney (protein concentration, 1-2 mg/ml) or 0.2 ml of solution containing defined single nephron segments was added to the tube. Incubation was carried out at 37 °C for 1 h with gentle shaking. After centrifugation at 3,000 × g for 5 min, the supernatant was discarded and the pellet was washed three times with 3.5 ml of isotonic solution containing 0.25% sucrose, 25 mm KCl, 5 mm MgCl2, 1 mm EDTA, 12 mm thiglycolate, 50 mm Tris-HCl, and 1 mg/ml of bovine serum albumin, pH 7.4. The pellet was resuspended in a hypotonic solution containing 0.3% KCl, 0.5 mm dithiothreitol, 1.5 mm EDTA, and 10 mM Tris-HCl, pH 7.4 (18, 19). The suspension was sonicated by a 15-s burst (Heat System Ultrasonics, Inc.), and the sonicate was centrifuged at 105,000 × g for 30 min in an L5-40 Beckman ultracentrifuge. Fractions (30 μl) were collected from the top of the tube using Buchler Auto-densi Flow, and were counted for radioactivity by scintillation spectrometry. [14C] Ovalbumin (3.7 S) was used as a gradient marker.

Receptors in the Isolated Single Nephron Segments—The uptake of 1,25(OH)2D3 in the presence or absence of unlabeled 1,25(OH)2D3 and the sucrose density gradient analysis were performed using microdissected PCT, MTAL, and collecting tubules in the same way as described above. Each incubation contained approximately 500-1000 mm total length of the specific tubule segment, and the data were expressed as femtomoles of 1,25(OH)2D3 internalized per 1000 mm tubular length.

Other Assays—Protein concentration of the tubular preparations was determined by the Lowry method (20).

RESULTS

Saturation Analysis—A typical experiment illustrating the 1,25(OH)2D3 uptake by the isolated tubular preparation which contains heterogenous tubule segments of the whole kidney is shown in Fig. 1. The difference between the total and the nonspecific uptake has been used to indicate the amount of 1,25(OH)2D3 specifically internalized. The Scatchard plot yielded a straight line, indicating a single class of binding sites with an apparent dissociation constant (Kd) of 0.54 nm and a total concentration of the binding site of 49 fmol/mg of protein. The value of Kd is similar to those obtained by other investigators for the kidney cells (7-9).

Displacement of Binding of 1,25(OH)2D3—Fig. 2 illustrates the effect of adding unlabeled 1,25(OH)2D3 on the 1,25(OH)2D3 uptake by a tubule preparation. The 1,25(OH)2D3 internalized by the tubular cells was displaced by unlabeled 1,25(OH)2D3 over a range of 10-2000 pg. However, in separate experiments, either unlabeled 25(OH)D3 or 24,25(OH)2D3 at 20 ng concentration displaced 1,25(OH)2D3 uptake by 36-40% (data not shown). When we performed similar experiments in the presence of human α-globulin fraction IV, we could eliminate the effects of these metabolites on the 1,25(OH)2D3 uptake (Fig. 3).

Density Gradient Analysis—The sedimentation properties of the tubular fragments were washed, sonicated, centrifuged, and the radioactivity of the 105,000 × g supernatant was counted. Nonspecific uptake was subtracted from total uptake to obtain specific uptake (①→②). B. Scatchard analysis of the specific uptake. Each point represents the mean of duplicate determinations.

![Fig. 1. Saturation analysis of 1,25(OH)2D3 uptake by isolated tubular preparations of the whole kidney. A. Aliquots of the suspension of tubular fragments (2.5 mg of protein/0.5 ml of medium) were incubated for 1 h at 37 °C with increasing concentrations of 1,25(OH)2D3 (i) alone (①→③) or in the presence of 200-fold excess of unlabeled 1,25(OH)2D3 (③→⑤). At the end of incubation, the tubular fragments were washed, sonicated, centrifuged, and the radioactivity of the 105,000 × g supernatant was counted. Nonspecific uptake was subtracted from total uptake to obtain specific uptake (①→②). B. Scatchard analysis of the specific uptake. Each point represents the mean of duplicate determinations.](image-url)
of the binding protein for 1,25(OH)$_2$D$_3$ in the 105,000 x g supernatant, examined by sucrose density gradient analysis, is shown in Fig. 4. The receptor-1,25(OH)$_2$[3H]D$_3$ complex was found to sediment at approximately 3.7 S in sucrose gradients prepared in the hypertonic buffer (Fig. 4A). However, when a tubular preparation was incubated with 25(OH)[3H]D$_3$, a radioactive peak appeared at 5.8 S, a value corresponding to the sedimentation constant for binding protein for 25(OH)D$_3$. This peak was displaced in the presence of a 200-fold excess of unlabeled 25(OH)D$_3$. These results again demonstrate that tubular cells can accumulate both 1,25(OH)$_2$D$_3$ and 25(OH)D$_3$; however, the proteins to which these metabolites bind are different.

1,25(OH)$_2$[3H]D$_3$ Uptake by Defined Single Nephron Segments—From the results described above, our assay system seems sensitive enough to localize 1,25(OH)$_2$D$_3$ uptake in the single nephron segments. Thus, we extended our methods attempting to quantify the amount of receptor in defined single nephron segments. We chose three nephron segments, i.e. PCT, MTAL, and collecting tubules. The MTAL is the segment where the nuclear uptake of 1,25(OH)$_2$D$_3$ has been detected by autoradiography. This segment is the easiest to dissect and collect in large quantity among the distal nephron segments, including cortical thick ascending limbs of Henle’s loop and distal convoluted tubules. The PCT is the segment where 25(OH)D$_3$-24-hydroxylase is localized; however, earlier autoradiographic data suggested little nuclear uptake of 1,25(OH)$_2$D$_3$. The collecting tubules is a segment where neither 1,25(OH)$_2$D$_3$ uptake nor 25(OH)D$_3$-24-hydroxylase have been detected. Because of the technical difficulty of dissecting sufficient quantities of these segments from the same animal during a reasonable period of time, i.e. a few hours, we studied only two segments, either PCT and MTAL or MTAL and collecting tubules, in any given experiment using the same rat. Results are summarized in Table I. The MTAL showed significant uptake of 1,25(OH)$_2$[3H]D$_3$, 20.8 ± 4.5 fmol/1000 mm tubular length/h. The PCT also showed significant uptake of 1,25(OH)$_2$[3H]D$_3$, quantities comparable to those obtained in the MTAL. By contrast, collecting tubules showed little or no uptake. In experiments 4 and 6, this segment showed a significant uptake of 1,25(OH)$_2$[3H]D$_3$, although this was much less than in the MTAL. In experiment 5, no significant uptake was

![Displacement curve of 1,25(OH)$_2$[3H]D$_3$ uptake.](image1.png)

**Fig. 2.** Displacement curve of 1,25(OH)$_2$[3H]D$_3$ uptake. Aliquots (0.1 ml) of suspension of tubule fragments (4 mg of protein) were incubated with 1,25(OH)$_2$[3H]D$_3$ (26 pg) alone and in the presence of 10-2000 pg of unlabeled 1,25(OH)$_2$D$_3$. Each point represents the mean of duplicate determinations.

![Competition by unlabeled vitamin D sterols for the 1,25(OH)$_2$[3H]D$_3$-binding sites in suspension of tubular preparations from whole kidney.](image2.png)

**Fig. 3.** Competition by unlabeled vitamin D sterols for the 1,25(OH)$_2$[3H]D$_3$-binding sites in suspension of tubular preparations from whole kidney. Aliquots (0.1 ml) of suspension of tubule fragments were incubated with 1,25(OH)$_2$[3H]D$_3$ (26 pg) alone and in the presence of 10-5200 pg of unlabeled 1,25(OH)$_2$D$_3$. Each point represents the mean of duplicate determinations.

| Nephron segments | Experiment | 1 | 2 | 3 | 4 | 5 | 6 | Mean ± S.E. |
|-----------------|------------|---|---|---|---|---|---|------------|
| PCT             |            | 44.2 | 29.3 | 16.1 | | | | 29.9 ± 8.1 |
| MTAL            |            | 19.4 | 18.9 | 11.6 | 34.9 | 17.8 | 22.6 | 20.8 ± 4.5 |
| CT              |            | 4.7 | 3.7 | 3.0 | | | | 3.5 ± 2.5 |

**Table I**

Uptake of 1,25(OH)$_2$[3H]D$_3$ by defined single nephron segments

Each defined single nephron segment was incubated for 1 h at 37 °C with 10 nm 1,25(OH)$_2$[3H]D$_3$ and with 200-fold excess, unlabeled 1,25(OH)$_2$D$_3$. At the end of incubation, nephron segments were rinsed, sonicated, centrifuged, and the radioactivity of 105,000 x g supernatant was counted. Specific uptake was obtained by subtracting nonspecific binding (with 200-fold unlabeled 1,25(OH)$_2$D$_3$ from total binding (without unlabeled 1,25(OH)$_2$D$_3$). Minimum detectable uptake was 0.6 fmol/1000 mm tubular length. CT, collecting tubules. The ranges of tubule lengths in the incubations were 527-1,154, 396-852, and 662-913 mm for the PCT, MTAL, and CT, respectively.

![Sucrose density gradient analysis of binding of 1,25(OH)$_2$D$_3$ and 23(OH)D$_3$ in the 105,000 x g supernatant of the sonicate of the tubule suspension.](image3.png)

**Fig. 4.** Sucrose density gradient analysis of binding of 1,25(OH)$_2$D$_3$ and 23(OH)D$_3$ in the 105,000 x g supernatant of the sonicate of the tubule suspension. A, binding of 1,25(OH)$_2$[3H]D$_3$ (○) and competition by 200-fold unlabeled 1,25(OH)$_2$D$_3$ (●). B, binding of 23(OH)[3H]D$_3$ (○) and competition by 200-fold unlabeled 23(OH)D$_3$ (●). Binding was established in tubule fragment suspensions incubated for 1 h at 37 °C. The arrow represents the position of the sedimentation marker, [13C] ovalbumin (3.7 S).
The present results clearly demonstrate that receptors for 1,25(OH)₂D₃ are localized both in the distal nephron and in the proximal nephron. The presence of the receptor in the distal nephron is consistent with earlier data of the exclusive localization of calcium binding protein inducible by vitamin D (12-14) and the nuclear uptake of 1,25(OH)₂[³H]D₃ in that part of nephron (11).

The presence of the receptor in the proximal nephron is at variance from autoradiographic data (11). The reason why autoradiography failed to demonstrate the nuclear uptake of 1,25(OH)₂[³H]D₃ in the proximal nephron is not known. It is possible that the time course of nuclear uptake of 1,25(OH)₂D₃ and/or the metabolism of the receptor-1,25(OH)₂D₃ complex may be different in the proximal nephron from those of the distal nephron. The 105,000 × g supernatant of the sonicates in hypertonic solution contains not only cytosol fraction but also some part of the chromatin-bound receptor fraction when measured separately in the tubular preparation from whole kidney; approximately 30% of the internalized 1,25(OH)₂[³H]D₃ was transferred to the nuclear fraction during 1 h of incubation at 37 °C. However, the quantities of the PCT or MTAL collected in one experiment are very limited and are not sufficient to allow measurement of the separate binding of 1,25(OH)₂D₃ in nuclear and cytosolic fractions. Nonetheless, the present data provide direct evidence for the presence of 1,25(OH)₂D₃ receptors in both the proximal and distal nephron of the rat. The results strongly support the thesis that two distinct proteins can be induced by 1,25(OH)₂D₃, 25(OH)D₃-24-hydroxylase, and a calcium-binding protein, each of which is present in the proximal or distal nephron, respectively.

Acknowledgment—We thank Dr. Jack W. Coburn for his critical reading of the manuscript.

1,25(OH)₂D₃ Receptors in Proximal and Distal Nephron 13431

FIG. 5. Sucrose density gradient analysis of 1,25(OH)₂D₃, binding in the 105,000 × g supernatant of the sonicated PCT and MTAL. A, binding of 1,25(OH)₂[³H]D₃ in 105,000 × g supernatant of sonicated MTAL (○) and competition by 200-fold unlabeled 1,25(OH)₂D₃ (●). B, binding of 1,25(OH)₂[³H]D₃ in the 105,000 × g supernatant of sonicated MTAL (○) and competition by 200-fold unlabeled 1,25(OH)₂D₃ (●). Binding was established in defined single nephron segments incubated for 1 h at 37 °C.
REFERENCES
1. DeLuca H. F., and Schnoes, H. K. (1976) Annu. Rev. Biochem. 45, 631–666
2. Norman, A. W. (1979) Vitamin D. The Calcium Homeostatic Steroid Hormone. Academic Press, New York
3. Lawson, D. E. M. (1978) Vitamin D Academic Press, New York
4. Brumbaugh, P. F., and Haussler, M. R. (1975) J. Biol. Chem. 250, 1588–1594
5. Taylor, A. N., and Wasserman, R. H. (1972) Am. J. Physiol. 223, 110–116
6. Puschett, J. B., Moranz, J., and Kurnick, W. S. (1972) J. Clin. Invest. 51, 373–385
7. Colston, K. W., and Feldman, D. (1979) J. Clin. Endocrinol. Metab. 48, 798–800
8. Chandler, J. S., Pike, J. W., and Haussler, M. R. (1979) Biochem. Biophys. Res. Commun. 90, 1057–1063
9. Christakos, S., and Norman, A. W. (1979) Biochem. Biophys. Res. Commun. 89, 56–63
10. Colston, K., and Feldman, D. (1980) J. Biol. Chem. 255, 7510–7513
11. Stumpf, W. E., Sar, M., Narbaitz, R., Reid, T. A., DeLuca, H. F., and Tanaka, Y. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1149–1153
12. Morrissey, R. L., Bucci, T. J., Empson, R. N., Jr., and Lufkin, E. D. (1975) Proc. Soc. Exp. Biol. Med. 149, 56–60
13. Rhoden, W. B., and Christakos, S. (1981) Endocrinology 109, 981–983
14. Christakos, S., Brunette, M. G., and Norman, A. W. (1981) Endocrinology 109, 322–324
15. Kawashima, H., Torikai, S., and Kurokawa, K. (1981) Proc Natl. Acad. Sci. U. S. A. 78, 1199–1203
16. Kurokawa, K., and Rasmussen, H. (1973) Biochim. Biophys. Acta 313, 17–31
17. Kawashima, H., Torikai, S., and Kurokawa, K. (1981) Nature (Lond.) 291, 327–329
18. Kream, B. E., Yamada, S., Schnoes, H. K., and DeLuca, H. F. (1977) J. Biol. Chem. 252, 4501–4505
19. Manolagas, S. C., and Deftos, L. F. (1980) Biochem. Biophys. Res. Commun. 85, 596–602
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
21. Tanaka, Y., Lorenc, R. S., and DeLuca, H. F. (1975) Arch. Biochem. Biophys. 171, 521–526
22. Henry, H. L. (1979) J. Biol. Chem. 254, 2722–2729