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PROPERTIES AND COMPARTMENTALIZATION OF THE TESTICULAR RECEPTOR FOR 1,25-DIHYDROXYVITAMIN D_3

FINN OLAV LEVY*, LARS EIKVAR, NICOLET H. P. M. JUTTE, ANNEKE FRØYSA, SVEIN MAGNE TVERMYR and VIDAR HANSSON
Institutes of Medical Biochemistry and Pathology, University of Oslo, Norway

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Summary—Adult rat testis contains a specific, high-affinity, low-capacity binding protein for 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) with properties similar to 1,25-$(OH)_2D_3$ receptors in other tissues. The receptor sediments at 3.5 ± 0.2 $S_{20,w}$ in high-salt sucrose density gradients, but aggregates in low-salt gradients. Binding of 1,25-$(OH)_2D_3$ was abolished by trypsin, but not by DNase or RNase. Binding was also heavily reduced by the sulfhydryl alkylating agent, *N*-ethylmaleimide, and by the mercurial reagent, mersalyl, showing that free, reduced SH-groups are necessary for hormone-binding activity. The receptor shows high affinity for 1,25-$(OH)_2D_3$ ($K_d = 3 \times 10^{-11}$ M), but low capacity ($N_{max} = 8$ fmol/mg protein) and is specific for 1,25-$(OH)_2D_3$ (Affinity: 1,25-$(OH)_2D_3$ > 1,24(R),25-$(OH)_3D_3$ > 25-OH-D_3 > 1α-OH-D_3 > 24(R),25-$(OH)_2D_3$ ≫ 17β-estradiol, testosterone, dexamethasone, R5020, progesterone). With 0.6 nM [^{3}H]1,25-$(OH)_2D_3$ and at 0°C, maximum specific binding was achieved after 4 h, and the occupied receptors were stable for more than 24 h. The dissociation of hormone–receptor complexes was temperature-dependent and very slow at low temperature ($t_{1/2}$ (0°C) ≫ 48 h). At 0°C, the second order association rate constant and the pseudo-first order dissociation rate constant were 2.7×10^7 M^{-1} min^{-1} and 2×10^{-5} min^{-1}, respectively. Receptors for 1,25-$(OH)_2D_3$ are present in similar amounts in isolated seminiferous tubules and interstitial tissue of adult rats. No specific binding of [^{3}H]1,25-$(OH)_2D_3$ could be detected in cultured immature Sertoli cells, cultured immature peritubular (myoid) cells or crude germ cells.

INTRODUCTION

The final metabolic activation of vitamin D_3 (formation of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$)† from 25-OH-D_3 by hydroxylation in 1α-position) takes place in the kidney [1–4]. It has been reported that nephrectomy causes a reduction in LH-stimulated cAMP formation in the testis, and that *in vivo* administration of 1,25-$(OH)_2D_3$ completely restores this defect [5]. There are also reports indicating specific binding of [^{3}H]1,25-$(OH)_2D_3$ in the testis [6–9]. However, the quantitative data are conflicting and 1,25-$(OH)_2D_3$ receptors in the testis are not extensively characterized. Furthermore, nothing is known about the distribution of 1,25-$(OH)_2D_3$ receptors between the interstitial and tubular compartments of the testis. For these reasons we have investigated further the properties and intratesticular distribution of this possible receptor for 1,25-$(OH)_2D_3$.

EXPERIMENTAL

Animals

Male Sprague–Dawley rats were raised on a standard laboratory diet and kept on a 12 h light–12 h dark cycle.

Chemicals

1α,25-Dihydroxy[26,27-methyl-^{3}H]cholecalciferol ([^{3}H]1,25-$(OH)_2D_3$) (158–160 Ci/mmol) was purchased from Amersham and New England Nuclear. Unlabelled vitamin D_3 analogues (1,25-$(OH)_2D_3$, 1,24(R),25-$(OH)_3D_3$, 25-OH-D_3, 1α-OH-D_3 and 24(R),25-$(OH)_2D_3$) were kindly provided by Dr W. Meier, Roche, Basel. Other unlabelled steroids as well as collagenase (type 1, no. C-0130), pancreatic deoxyribonuclease (925 Kunitz units/mg protein, essentially salt-free, substantially free of RNase), pancreatic ribonuclease A (70 Kunitz units/mg protein, essentially protease and salt free), bovine serum albumin (BSA), *N*-ethylmaleimide, mersalyl acid, sodium molybdate (Na_2MoO_4), Triton-X-100, EDTA and Trizma base were all obtained from Sigma Chemical Company. Hydroxylapatite (Bio-Gel HTP) was obtained from Bio-Rad. Trypsin ("1:250") was obtained from DIFCO; dithiothreitol

*Correspondence to: Finn Olav Levy, Institute of Medical Biochemistry, University of Oslo, P.O. Box 1112 Blindern, N-0317 Oslo 3, Norway.

†The abbreviations used are: 1,25-$(OH)_2D_3$ = 1α,25-dihydroxyvitamin D_3; 1,24(R),25-$(OH)_3D_3$ = 1α,24(R),25-trihydroxyvitamin D_3; 25-OH-D_3 = 25-hydroxyvitamin D_3; 1α-OH-D_3 = 1α-hydroxyvitamin D_3; 24(R),25-$(OH)_2D_3$ = 24(R),25-dihydroxyvitamin D_3; KTEDMo = 10 mM Tris–HCl, 300 mM KCl, 1.5 mM EDTA, 1 mM DTT, 10 mM Na_2MoO_4, pH 7.4 at 23°C; PBS = phosphate-buffered saline; DCC = dextran-coated charcoal; DBP = serum vitamin D binding protein; R5020 = 17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione; EDTA = ethylene diamine tetraacetic acid; DTT = dithiothreitol; BSA = bovine serum albumin; HBSS = Hanks' balanced salt solution.

(DTT) from Calbiochem; Hanks' balanced salt solution (HBSS) from GIBCO; dextran (T-70) from Pharmacia; charcoal from BDH; KCl from Merck; sucrose from Riedel de Haën; ovalbumin and chymotrypsinogen (unlabelled) from Serva, Heidelberg and ^{14}C-labelled ovalbumin and carbonic anhydrase were from New England Nuclear.

Cytosol preparation

All tissues were homogenized or sonicated at 0°C in high-salt buffer containing 10 mM Tris–HCl, 300 mM KCl, 1.5 mM EDTA, 1 mM DTT and 10 mM Na_2MoO_4 (KTEDMo buffer), pH 7.4 at 23°C. The homogenates/sonicates were left at 0°C for 30–60 min to extract nuclear receptors followed by centrifugation at 105,000 *g* for 60 min at 1°C. The supernatant (without the lipid layer) was used as the cytosol.

Whole testis cytosol was prepared from decapsulated testes which were rinsed in ice-cold phosphate-buffered saline (PBS). The testes were then homogenized in a Dounce all glass homogenizer in 3 vol of KTEDMo by 10 strokes with pestle A (loose-fitting) and 10 strokes with pestle B (tight-fitting). Seminiferous tubules and interstitial tissue were separated by wet dissection [10] in HBSS at room temperature. The isolated tissues were centrifuged at 120 *g* for 5 min at 0°C and the pellets were homogenized in ice-cold KTEDMo.

Sertoli cells were obtained from 19-day old rats by enzyme treatment (trypsin, collagenase and DNase) followed by culture for 5 days [11] after which the cells were scraped from the dishes and sonicated for 3 × 5 s in KTEDMo.

Peritubular cells were isolated from 19-day old rats by the method of Hutson and Stocco[12] and cultured and sonicated as the Sertoli cells. Crude germ cells were obtained from 32-day old rats by enzyme treatment (collagenase and trypsin) as described in [13] and sonicated in KTEDMo as described above.

Intestinal cytosol was prepared by rinsing the whole intestine with ice-cold PBS followed by scraping of the mucosa from the muscle layer with a glass slide at 0°C. The mucosa was then washed three times in 7–8 vol ice-cold PBS by centrifugation at 200 *g* for 5 min before sonication in KTEDMo as described above.

The protein content of each cytosol was measured by the method of Lowry *et al.*[14].

Binding assays

Cytosol was incubated with [^{3}H]1,25-$(OH)_2D_3$ without additions or in the presence of cold steroids at 0°C for the times indicated in the figure legends. Because some of the tracer adsorbed to the incubation tubes, the final concentration of [^{3}H]1,25-$(OH)_2D_3$ in each experiment was calculated from the radioactivity counted in aliquots of the incubation mixtures or parallel incubations. Bound and free steroids were separated by hydroxylapatite assay essentially according to [15] or sucrose gradient centrifugation essentially according to [16]. Details of the methods are described below.

Hydroxylapatite assay

A 50% slurry (v/v) of hydroxylapatite was prepared in either 10 mM potassium phosphate buffer containing 100 mM KCl, pH 7.5 or KTEDMo. Labelled cytosol (100 or 200 μl) was incubated with 500 μl hydroxylapatite slurry for 15 min at 0°C with frequent blending on a Vortex mixer. The hydroxylapatite was then washed three times with 2.0 ml KTEDMo containing 0.5% Triton-X-100 by centrifugation at 2000 *g* for 5 min. The final hydroxylapatite pellet was extracted with 2.0 ml ethanol once or with 1.0 ml ethanol twice for at least 10 min at room temperature. After centrifugation, the supernatants were decanted into counting vials and counted for radioactivity in an LKB Wallac liquid scintillation counter.

Sucrose density gradient analysis

4.2 ml linear 5–20% (w/v) sucrose density gradients were prepared with a Buchler gradient mixer. Labelled cytosol (200 μl) was incubated with 25 μl DCC (dextran-coated charcoal; 5% charcoal, 0.5% dextran T-70 in KTEDMo) at 0°C for 15 min. After centrifugation at 2000 *g* for 10 min, 200 μl of the supernatant was carefully layered on the gradient, which was then centrifuged as described in the legend to each figure. After the ultracentrifugation, fractions of eight drops each were collected from the bottom and counted for radioactivity.

RESULTS

1,25-$(OH)_2D_3$ receptors in high-salt cytosol from whole testis

Figure 1 shows binding of [^{3}H]1,25-$(OH)_2D_3$ in high-salt cytosol from whole testis examined by sucrose gradient centrifugation. High-salt cytosol from whole testis was incubated with 0.4 nM [^{3}H]1,25-$(OH)_2D_3$ alone or in the presence of a 200-fold excess of unlabelled 1,25-$(OH)_2D_3$. As seen from Fig. 1A, there was a large peak of specific binding of 1,25-$(OH)_2D_3$ with a sedimentation coefficient of approx 5–6 S.

Figures 1B and 1C show sucrose gradient profiles from the same experiment. However, in this case the samples have been adsorbed by dextran-coated charcoal for 15 min prior to sucrose gradient centrifugation. As seen from Fig. 1B, the peak of binding in the 5–6 S region was decreased from approx 2000 to approx 800 cpm and now a distinct peak of binding became apparent in the 3.5 S region of the sucrose gradient.

In Fig. 1C, the cytosol has been labelled with [^{3}H]1,25-$(OH)_2D_3$ with and without unlabelled 1,25-$(OH)_2D_3$ as in Figs 1A and 1B. However, after labelling, the samples were further incubated for 1 h

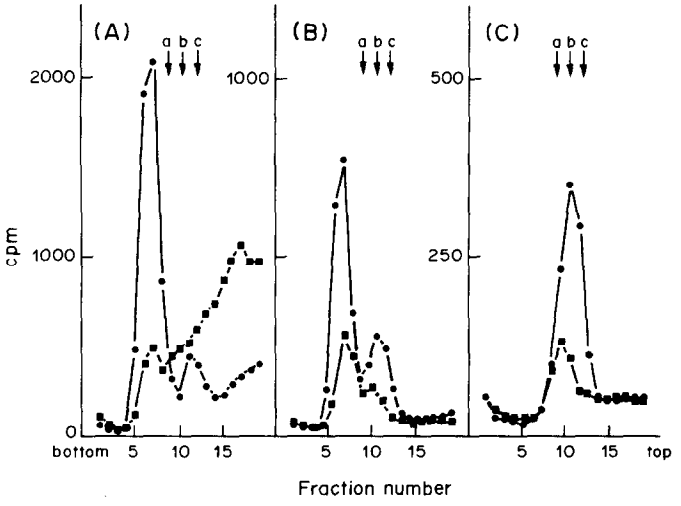

Fig. 1. Sucrose gradient analysis of [^{3}H]1,25-$(OH)_2D_3$ binding in high-salt cytosol from whole testis. Cytosol (7.2 mg protein/ml) was incubated for 18 h at 0°C with 0.4 nM [^{3}H]1,25-$(OH)_2D_3$ with (■——■) and without (●——●), a 200-fold excess of unlabelled 1,25-$(OH)_2D_3$. A: After labelling, 200 μl aliquots of labelled cytosol were layered directly on sucrose gradients. B,C: After labelling, the incubations were continued for 1 h at 0°C with 100 nM (final concentration) of unlabelled 25-OH-D_3 (C) or vehicle only (B). Free steroids were then adsorbed with DCC for 15 min at 0°C and 200 μl aliquots of the supernatants were layered on linear sucrose gradients. The gradients were centrifuged in a Beckman L8-80 ultracentrifuge with a SW 50.1 Swinging Bucket Rotor at 49,000 rpm (225,000 g_{av}), 1°C, for 16 h. BSA (4.4 S,a), ovalbumin (3.7 S,b) and carbonic anhydrase (3.0 S,c) were used as external sedimentation marker proteins.

at 0°C in the presence of an excess (100 nM) of unlabelled 25-OH-D_3. Free steroids were then adsorbed by dextran-coated charcoal before sucrose gradient centrifugation. This treatment completely eliminated binding of [^{3}H]1,25-$(OH)_2D_3$ to the 5–6 S peak without reducing the specific binding to the 3.5 S peak.

1,25-$(OH)_2D_3$ receptors in seminiferous tubules

Figure 2 shows experiments in which high-salt cytosol from isolated seminiferous tubules was incubated with [^{3}H]1,25-$(OH)_2D_3$ (0.26 nM) and increasing concentrations of unlabelled 1,25-$(OH)_2D_3$ (0.1, 0.2, 0.4, 0.8 or 1000 nM). The insert in this figure shows percentage specific binding in the 3.5 S region of the gradient as a function of the concentration of cold 1,25-$(OH)_2D_3$ added. As seen from the figure, there was a concentration dependent decrease in specific binding to the 3.5 S moiety, and the concentration of 1,25-$(OH)_2D_3$ required for 50% inhibition of specific binding was approx 0.3 nM. As also should be noted from this figure, the isolation procedure of the seminiferous tubules completely eliminated the binding in the 5–6 S region of the sucrose gradient.

Association, dissociation and stability

In Fig. 3, the time-course of binding of [^{3}H]1,25-$(OH)_2D_3$ to the receptor in seminiferous tubules is shown at three different concentrations of radioactive steroid. Maximal specific binding was achieved after 4 h when the concentration of [^{3}H]1,25-$(OH)_2D_3$ was 0.64 nM. At lower hormone concentrations, the time required for equilibration was longer.

Figure 4 shows the rate of dissociation for the 1,25-$(OH)_2D_3$ receptor in seminiferous tubules at two different temperatures (0 and 16°C). It also examines the stability of the steroid receptor complexes at the two temperatures. At 0°C, the dissociation rate was very slow ($t_{\frac{1}{2}} \gg 48$ h), and the occupied receptors appeared to be stable for more than 24 h. At 16°C, the dissociation clearly followed pseudo-first order kinetics with a half-life ($t_{\frac{1}{2}}$) of 8.2 h, and also at this temperature the occupied receptors were stable for more than 24 h. At 29°C (results not shown), the dissociation was rapid ($t_{\frac{1}{2}} \approx \frac{1}{2}$ h) and in addition there was a significant destruction of hormone receptor complexes during the incubation.

Association (k_1) and dissociation (k_{-1}) rate constants were calculated from these kinetic studies. The k_1 was calculated to be $2.70 \pm 0.15 \times 10^7\ M^{-1}\ min^{-1}$ at 0°C, whereas the pseudo-first order dissociation rate constant, k_{-1}, was $1.4 \times 10^{-3}\ min^{-1}$ at 16°C and approx $2 \times 10^{-5}\ min^{-1}$ at 0°C. The rate constants at 0°C indicate an equilibrium constant of dissociation (K_d) in the order of 10^{-12} M ($K_d = k_{-1}/k_1 \approx 1 \times 10^{-12}$ M).

Binding affinity and capacity

Cytosol from seminiferous tubules was incubated with increasing concentrations of [^{3}H]1,25-$(OH)_2D_3$ (0.006–1.0 nM) in the absence (total binding) or presence (non-specific binding) of a 200-fold excess of unlabelled 1,25-$(OH)_2D_3$. Total, non-specific and specific binding (difference) were estimated with both the hydroxylapatite assay and sucrose gradient analysis. As shown in Figs 5A and C, both methods yielded quite similar results. A saturable specific

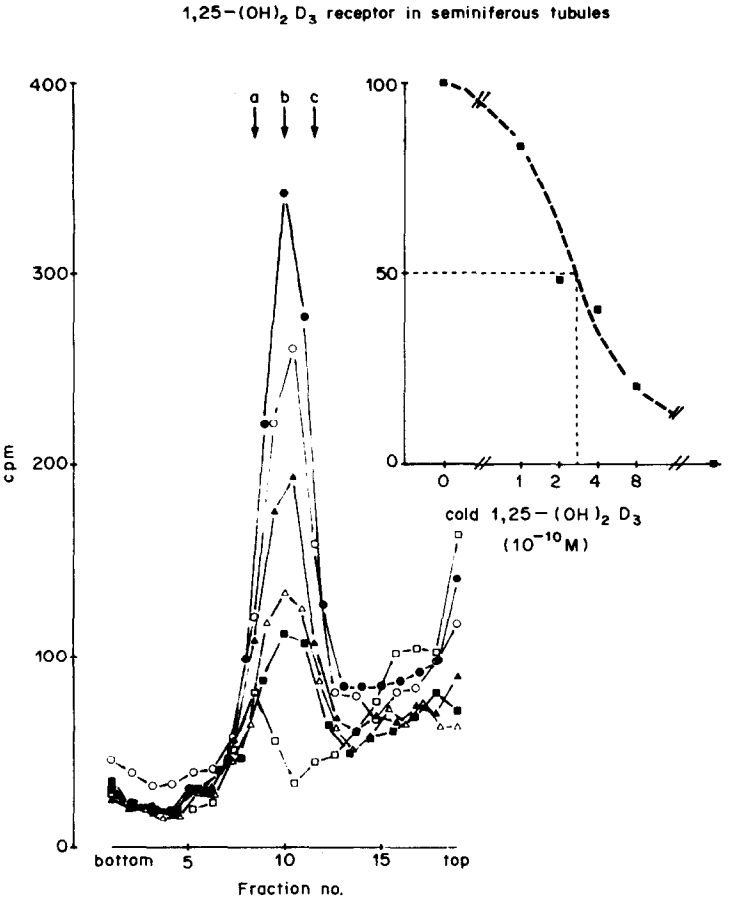

Fig. 2. Sucrose gradient analysis of [^{3}H]1,25-$(OH)_2D_3$ binding in high-salt cytosol from seminiferous tubules. Cytosol (6.9 mg protein/ml) was incubated for 18.5 h at 0°C with 0.26 nM [^{3}H]1,25-$(OH)_2D_3$ alone (●——●), or with 0.26 nM [^{3}H]1,25-$(OH)_2D_3$ plus the following concentrations of unlabelled 1,25-$(OH)_2D_3$: 0.1 nM (○——○), 0.2 nM (▲——▲), 0.4 nM (△——△), 0.8 nM (■——■) or 1000 nM (□——□) (non-specific binding). The gradients were centrifuged as described in the legend to Fig. 1, and the same sedimentation marker proteins were used.

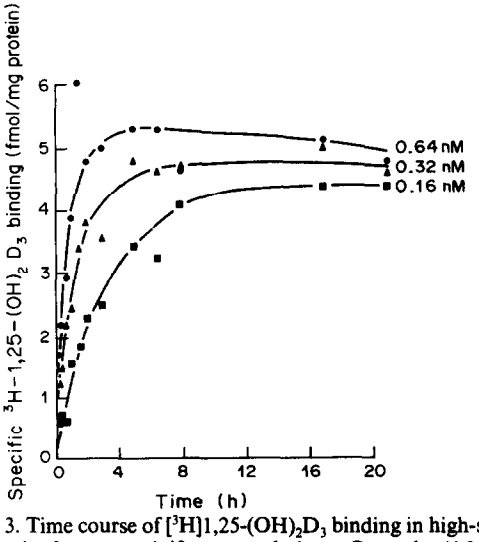

Fig. 3. Time course of [^{3}H]1,25-$(OH)_2D_3$ binding in high-salt cytosol from seminiferous tubules. Cytosol (4.3 mg protein/ml) was incubated with three different concentrations of [^{3}H]1,25-$(OH)_2D_3$ at 0°C. One-hundred μl samples (triplicates) were removed at various time intervals and assayed for specific binding using the hydroxylapatite assay.

binding component was demonstrated using both methods, whereas the non-specific binding increased linearly with the concentrations of [^{3}H]1,25-$(OH)_2D_3$ used. Scatchard analysis [17] of the specific binding calculated from the hydroxylapatite assay (Fig. 5B) yielded a straight line (correlation coefficient, $r = -0.98$), indicating a single class of non-interacting binding sites. The equilibrium dissociation constant, K_d, calculated from the slope of this line was 2.7×10^{-11} M. The number of binding sites (N_{max}) calculated from the X-intercept of the Scatchard plot was 8.3 fmol/mg protein. The sucrose gradient analysis (Figs 5C and D) yielded similar results; K_d and N_{max} values were 3.8×10^{-11} M and 6.0 fmol/mg protein, respectively.

Specificity of 1,25-$(OH)_2D_3$ receptors in seminiferous tubules

Cytosol from seminiferous tubules was incubated with [^{3}H]1,25-$(OH)_2D_3$ alone or in the presence of increasing concentrations of various unlabelled vitamin D_3 analogues or a single concentration (1.0 μM) of other unlabelled steroids. Specific binding was

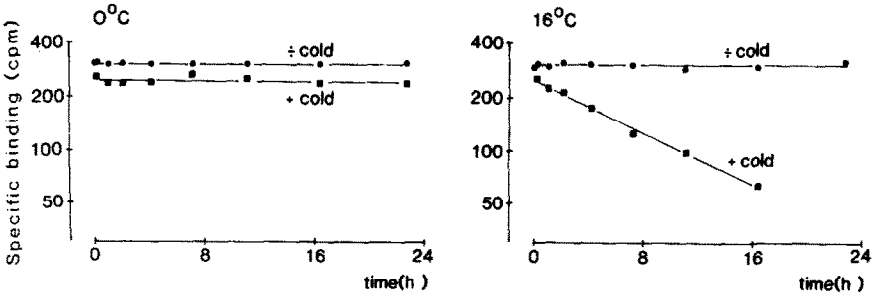

Fig. 4. Rate of dissociation for $1,25\text{-}(OH)_2D_3$ receptors in high-salt cytosol from seminiferous tubules. Cytosol (4.7 mg protein/ml) was pre-incubated at 0°C for 15 h with 0.4 nM [^{3}H]$1,25\text{-}(OH)_2D_3$ with or without 100 nM unlabelled $1,25\text{-}(OH)_2D_3$. At time zero, the fraction preincubated in the absence of unlabelled ligand was divided into two parts, one receiving an excess of unlabelled $1,25\text{-}(OH)_2D_3$ (100 nM final), the other receiving the solvent (ethanol/buffer) (control for receptor degradation). The rate of dissociation was determined at both 0 and 16°C. At the times indicated, 100 μl-aliquots from each incubation were assayed in triplicate for specific binding of [^{3}H]$1,25\text{-}(OH)_2D_3$ using the hydroxylapatite assay. – cold: Control for receptor degradation. + cold: Excess non-radioactive $1,25\text{-}(OH)_2D_3$.

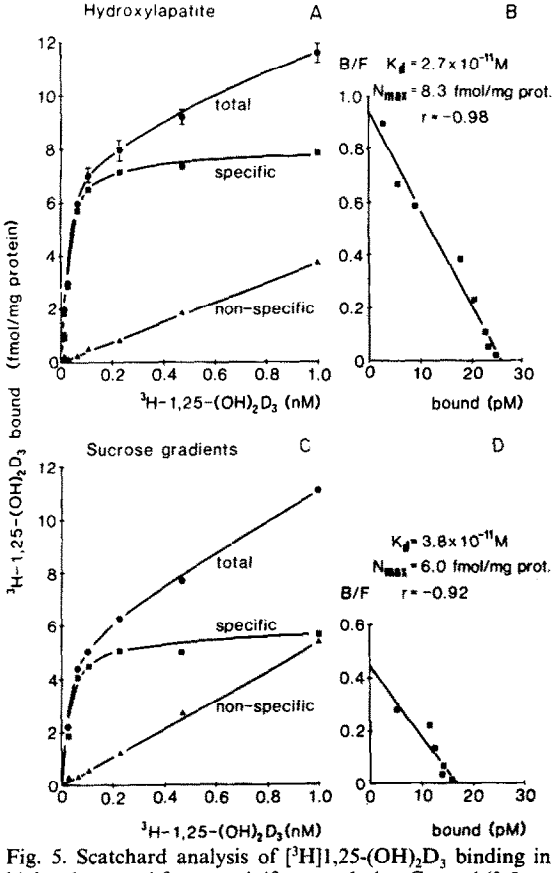

Fig. 5. Scatchard analysis of [^{3}H]$1,25\text{-}(OH)_2D_3$ binding in high-salt cytosol from seminiferous tubules. Cytosol (3.5 mg protein/ml) was incubated at 0°C for 15 h (hydroxylapatite assay) or 19 h (sucrose gradients) with increasing concentrations of [^{3}H]$1,25\text{-}(OH)_2D_3$ (hydroxylapatite assay, 0.006–1.0 nM; sucrose gradient analysis, 0.24–1.0 nM) with and without a 200-fold excess of unlabelled $1,25\text{-}(OH)_2D_3$. A; 100 μl-aliquots (quadruplicates) from each incubation tube were assayed for [^{3}H]$1,25\text{-}(OH)_2D_3$ binding with the hydroxylapatite assay. B; Scatchard analysis of the data depicted in Fig. 5A. C; An aliquot from each incubation was assayed for [^{3}H]$1,25\text{-}(OH)_2D_3$ binding by sucrose gradient analysis. The gradients were centrifuged as described in the legend to Fig. 1, and the area below the specific binding peak at each concentration of [^{3}H]$1,25\text{-}(OH)_2D_3$ was taken as the amount of [^{3}H]$1,25\text{-}(OH)_2D_3$ bound. D; Scatchard analysis of the data depicted in Fig. 5C.

estimated using both the hydroxylapatite assay and sucrose gradient analysis. As shown in Fig. 6, the receptor is relatively specific for $1,25\text{-}(OH)_2D_3$. The ability to compete with [^{3}H]$1,25\text{-}(OH)_2D_3$ for the binding sites decreased in the following order: $1,25\text{-}(OH)_2D_3 > 1,24(R),25\text{-}(OH)_3D_3 > 25\text{-OH-}D_3 > 1\alpha\text{-OH-}D_3 > 24(R),25\text{-}(OH)_2D_3$. Other steroids (17$\beta$-estradiol, testosterone, dexamethasone, progesterone and R5020) showed no ability to compete with [^{3}H]$1,25\text{-}(OH)_2D_3$ for the binding sites.

Aggregation of $1,25\text{-}(OH)_2D_3$ receptors at low ionic strength

Cytosol from seminiferous tubules, incubated with [^{3}H]$1,25\text{-}(OH)_2D_3$ in the absence (total binding) and presence (non-specific binding) of excess unlabelled $1,25\text{-}(OH)_2D_3$ were layered on sucrose gradients, containing 5–20% sucrose in KTEDMo (0.3 M KCl) or in the same buffer without KCl. The $1,25\text{-}(OH)_2D_3$ receptors aggregated in the low-salt gradients (results not shown) similar to the aggregation of $1,25\text{-}(OH)_2D_3$ receptors in other tissues [18, 19].

Chemical nature of the receptor

High-salt cytosols from seminiferous tubules and from whole testis were subjected to enzymatic treatment with trypsin, DNase and RNase (Table 1). As with the receptors in intestinal mucosa [20], trypsin completely eliminated binding of [^{3}H]$1,25\text{-}(OH)_2D_3$ to the receptor, whereas DNase and RNase were without effect. Furthermore, the sulfhydryl alkylating agent, *N*-ethylmaleimide, and the mercurial reagent, mersalyl, both eliminated binding of [^{3}H]$1,25\text{-}(OH)_2D_3$ to the receptor, indicating that free, reduced SH-groups (cysteine-residues) are necessary for the hormone binding activity.

Compartmentalization of the testicular $1,25\text{-}(OH)_2D_3$ receptors

The results shown in Table 2, indicate that both the seminiferous tubules and the interstitial tissue from adult rats contain receptors for $1,25\text{-}(OH)_2D_3$. The

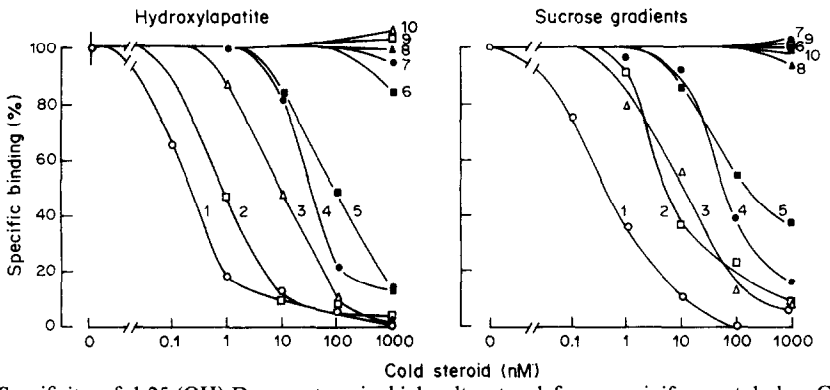

Fig. 6. Specificity of 1,25-$(OH)_2D_3$ receptors in high-salt cytosol from seminiferous tubules. Cytosol (2.7 mg protein/ml) was incubated at 0°C for 5 h with 0.45 nM [^{3}H]1,25-$(OH)_2D_3$ alone or with 0.45 nM [^{3}H]1,25-$(OH)_2D_3$ plus the indicated concentration of unlabelled steroid. The amount of bound [^{3}H]1,25-$(OH)_2D_3$ was estimated both with the hydroxylapatite assay (triplicates) and with sucrose gradient analysis. The sucrose gradients were centrifuged as described in the legend to Fig. 1. The unlabelled steroids were: 1: 1,25-$(OH)_2D_3$, 2: 1,24R,25-$(OH)_3D_3$, 3: 25-OH-D_3, 4: 1α-OH-D_3, 5: 24R,25-$(OH)_2D_3$, 6: Progesterone, 7: Dexamethasone, 8: R5020, 9: 17β-Estradiol, 10: Testosterone.

amount of specific binding, calculated per mg protein, was very similar in the two compartments. In contrast, specific binding of [^{3}H]1,25-$(OH)_2D_3$ was not detected in similar cytosol fractions from cultured Sertoli cells or peritubular cells from immature (19-day old) rats, nor in crude germ cells from 32-day old rats.

DISCUSSION

This study shows the presence of a receptor for 1,25-$(OH)_2D_3$ in high-salt cytosol from the testis of adult rats, with properties (affinity, specificity, sedimentation coefficient, binding kinetics and stability) similar to the receptors for 1,25-$(OH)_2D_3$ described in

Table 1. Chemical nature of the 1,25-$(OH)_2D_3$ receptor in high-salt cytosol from seminiferous tubules and whole testis

Cytosol from	Preincubation with	Specific binding (% of control)
Seminiferous tubules	Control (Tris-HCl)	100
	Trypsin	1
	DNase	63
	RNase	97
Whole testis	Control (Tris-HCl)	100
	Trypsin	0
	DNase	74
	RNase	104
	N-ethylmaleimide	27
	Mersalyl	21

Cytosol (180 μl, seminiferous tubules, 8.0 mg protein/ml; whole testis, 4.8 mg protein/ml) was preincubated at 0°C for 30 min with either 50 μl 50 mM Tris-HCl, pH 7.4 (23°C) (control), 50 μl Tris-HCl containing 1 mg/ml of trypsin, DNase or RNase or 50 μl Tris-HCl containing 50 mM *N*-ethylmaleimide or 5.0 mM mersalyl. After the preincubation, [^{3}H]1,25-$(OH)_2D_3$ (seminiferous tubules, 0.2 nM; whole testis, 0.1 nM) was added with or without 1000-fold excess of unlabelled 1,25-$(OH)_2D_3$ and the incubation was continued for 20 h at 0°C before estimation of specific binding with the hydroxylapatite assay. The specific binding in the control incubation was taken as 100% (seminiferous tubules, 4.9 fmol/mg protein; whole testis, 2.2 fmol/mg protein).

Table 2. Distribution of the testicular 1,25-$(OH)_2D_3$ receptor between the different compartments of the testis

	Hydroxylapatite assay		Sucrose gradient analysis	
Seminiferous tubules	6.4 ± 1.6	(n = 18)	8.6 ± 2.5	(n = 10)
Interstitial tissue	6.0 ± 0.6	(n = 3)	9.8	(n = 1)
Whole testis	6.5 ± 1.1	(n = 6)	5.4 ± 1.6	(n = 6)
Sertoli cells (19 days)	0.9	(n = 1)	<0.5*	(n = 3)
Germs cells (32 days)	ND		<0.5*	(n = 1)
Pertibular cells (19 days)	ND		<0.5*	(n = 1)

The amount of 1,25-$(OH)_2D_3$ receptor in each compartment was estimated by either Scatchard analysis or single-point estimate of specific [^{3}H]1,25-$(OH)_2D_3$ binding in high-salt cytosol at 0°C. Mean ± SD of n different cytosol preparations. ND: not determined.

*Detection limit.

other tissues [1–4, 21–22]. The receptor was present both in isolated seminiferous tubules and in the interstitial tissue of adult rats.

The first demonstration of specific binding of 1,25-$(OH)_2D_3$ in the testis was by Kream *et al.*[6]. They demonstrated a small peak of specific binding in the 3.2 S region of sucrose gradients, but this activity was not further characterized. The major binding of 1,25-$(OH)_2D_3$ in the testis cytosol was to the serum vitamin D binding protein (DBP), which, after forming a complex with actin, sediments in the 5–6 S region of the sucrose gradients [23–26]. Binding of [^{3}H]1,25-$(OH)_2D_3$ to the DBP in the testis cytosol can to some extent be eliminated by charcoal adsorption (Fig. 1B). However, much better removal of [^{3}H]1,25-$(OH)_2D_3$ binding to serum DBP was achieved by differential displacement of DBP bound radioactivity after labelling (Fig. 1C). The fact that dissociation of [^{3}H]1,25-$(OH)_2D_3$ from the serum DBP is rapid ($t_{\frac{1}{2}}(0°C) \lesssim 5$ min, results not shown) whereas that from the receptor is very slow ($t_{\frac{1}{2}}(0°C) \gg 48$ h) makes it possible to remove binding to the serum DBP without affecting that to the receptor.

Merke *et al.*[7] also demonstrated binding of [^{3}H]1,25-$(OH)_2D_3$ to a 3.5 S component when analysed on high-salt sucrose density gradients. The amount of specific binding of [^{3}H]1,25-$(OH)_2D_3$ they observed was more than 10 times higher than that found in the present study (82 and 6–8 fmol/mg protein, respectively). Furthermore, their estimation of binding affinity ($K_d = 2.6 \times 10^{-10}$ M) by Scatchard analysis was one order of magnitude lower than that observed in the present study ($K_d = 3 \times 10^{-11}$ M). Repeated attempts to reproduce their studies were not successful. The reasons for the apparent discrepancies remain to be established.

Walters *et al.* have also suggested the presence of 1,25-$(OH)_2D_3$ receptors in the testes[8, 9]. They recommended a chromatin preparation technique to avoid the problem with binding to the serum DBP. The amount of receptors demonstrated with this technique was approx 10 fmol/g testis [9]. Assuming that 1 g of testis tissue contains approx 40 mg of cytosol protein, this is 0.3 fmol/mg cytosol protein, a figure which is 20–25 times lower than that observed in the present study (Fig. 5).

Isolation of seminiferous tubules by wet dissection also proved a convenient way of eliminating binding to the contaminating serum DBP, and in this preparation specific binding of 1,25-$(OH)_2D_3$ was observed only in the 3.5 S region of the sucrose gradient (Fig. 2). The binding was saturable with sub-nanomolar concentrations of [^{3}H]1,25-$(OH)_2D_3$ and had a sedimentation coefficient very similar to the 1,25-$(OH)_2D_3$ receptors in the intestinal mucosa (results not shown).

Calculation of the association rate constant (k_1) for [^{3}H]1,25-$(OH)_2D_3$ binding to the receptor in the seminiferous tubules was compatible with a second-order reaction [27]. At 0°C and using three different concentrations of [^{3}H]1,25-$(OH)_2D_3$ (Fig. 3), the k_1 value was found to be $2.70 \pm 0.15 \times 10^7\ M^{-1}\ min^{-1}$ (SD, $n = 3$). This is similar to the k_1 value of $1.7 \pm 0.7 \times 10^7\ M^{-1}\ min^{-1}$ ($n = 2$) reported by Wecksler *et al.*[20] for the 1,25-$(OH)_2D_3$ receptor in rat intestinal mucosa, and represents another indication of the similarity between receptors in various tissues. Dissociation studies (Fig. 4) yielded a pseudo-first order dissociation rate constant, k_{-1}, of about $2 \times 10^{-5}\ min^{-1}$ at 0°C, whereas at 16°C the k_{-1} was $1.4 \times 10^{-3}\ min^{-1}$.

Feldman *et al.*[19] reported that in high-salt cytosol from rat intestinal mucosa, specific binding of [^{3}H]1,25-$(OH)_2D_3$ (1.3 nM) was optimal after 5 h at 0°C, and then decreased to about 65% of the maximal value after 24 h. We did not observe a similar decrease in specific binding with time in high-salt cytosol from seminiferous tubules (Figs 3 and 4).

The association rate studies furthermore showed that an 8–16 h incubation was required to obtain equilibrium at the lowest concentrations of [^{3}H]1,25-$(OH)_2D_3$ (Fig. 3). For the saturation analysis we therefore used a 15 h incubation period (Fig. 5). Scatchard analysis of the specific binding yielded a K_d value of 2.7×10^{-11} M at 0°C (Figs 6A and B, hydroxylapatite assay). Sucrose gradient analysis (Figs 6C and D) gave similar results ($K_d = 3.8 \times 10^{-11}$ M at 0°C).

It has been previously observed for the 1,25-$(OH)_2D_3$ receptors [20, 22], and also for other steroid receptors [28], that the ratio of the rate constants (k_{-1}/k_1) has been in poor agreement with the apparent K_d calculated by Scatchard analyses. This has also been suggested to be indicative of a complex binding mechanism [22, 28], but it may also be due to other non receptor binding proteins present in the crude cytosol preparations [21]. Our results also indicated some discrepancy between the K_d calculated from the rate constants ($k_{-1}/k_1 \approx 1 \times 10^{-12}$ M) and the K_d calculated from the Scatchard plots (2.7×10^{-11} M).

The low concentration of receptor in the seminiferous tubules may suggest that the receptor is localized in a subpopulation of cells. However, no specific binding of [^{3}H]1,25-$(OH)_2D_3$ was detected in crude germ cells, nor in cultured Sertoli cells and peritubular cells from immature rats. It is not known whether the lack of receptors in cultured tubular cells is due to the age of the animals or to the culture conditions.

Halloran and deLuca[29] have found that the concentration of 1,25-$(OH)_2D_3$ receptors in the rat intestinal mucosa is very low prior to day 14 post partum, compared with the concentration at day 28 post partum and in the adult rat. This suggests an age-dependent appearance of this receptor in the intestinal mucosa. It remains to be determined whether age dependent changes may take place in the testis.

In conclusion, both the seminiferous tubules and the interstitial tissue in testes of adult rats contain a receptor for 1,25-$(OH)_2D_3$. These receptors are undetectable in cultured cells isolated from immature (19-day old) rats, and their possible physiological importance remains to be established.

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