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APPEARANCE OF THE RAT TESTICULAR RECEPTOR FOR CALCITRIOL (1,25-DIHYDROXYVITAMIN D_3) DURING DEVELOPMENT

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Summary—In the present study we have examined the developmental changes in the concentration of receptors for calcitriol in high-salt cytosol from the rat testis. Receptors for calcitriol were undetectable (< 0.4 fmol/mg protein) until day 24, after which there was a rapid increase to reach adult levels (6–8 fmol/mg protein) between day 50–60. The lack of receptors in high-salt cytosol from the immature rat testis is not due to degrading enzymes, since cytosols prepared from the combination of equal volumes of testis homogenates from immature and adult rats had binding levels exactly half of that found in "adult controls". Furthermore, the increase in specific binding of [^{3}H]calcitriol during development is due to an increase in the number of receptor sites, and is not due to a change in the apparent affinity of the receptors ($K_d \approx 1 \times 10^{-11}$ M at 0°C). These results may explain why we previously were unable to demonstrate calcitriol receptors in cultured Sertoli cells and peritubular cells isolated from 19-day old rats. Furthermore, they indicate that calcitriol may be of minor importance for testicular function in the immature rat. The role of calcitriol in the pubertal and adult testis remains to be established.

INTRODUCTION

The rat testis contains a receptor for calcitriol (1,25-dihydroxyvitamin D_3)* [1–3]. This receptor is present both in seminiferous tubules and interstitial tissue of adult rats, but was not found in cultured Sertoli cells or peritubular (myoid) cells isolated from immature (19-day old) rats [3]. It is not known whether this lack of calcitriol receptors in cultured cells from immature rats is due to the culture conditions or to the age of the animals.

Therefore, we examined the concentration of calcitriol receptors in high-salt testis cytosols from rats of various ages (19–272 days), in order to determine whether there are quantitative changes in these receptors during development. This study shows that calcitriol receptors were undetectable (< 0.4 fmol/mg protein) in the rat testis before day 24 of age, and that the receptor concentration increased gradually to reach adult levels (6–8 fmol/mg protein) around day 50–60. Some of these results have been presented in a preliminary report [4].

*The abbreviations used are: calcitriol = 1,25-$(OH)_2D_3$ = 1α,25-dihydroxyvitamin D_3 = (5Z,7E)-(1S,3R)-9,10-seco-5,7,10(19)-cholestatriene-1,3,25-triol; calcidiol = 25-OH-D_3 = 25-hydroxyvitamin D_3 = (5Z,7E)-(3S)-9,10-seco-5,7,10(19)-cholestadiene-3,25-diol; 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; EDTA = ethylene diamine tetraacetic acid; DTT = dithiothreitol; BSA = bovine serum albumin; HBSS = Hanks' balanced salt solution; LH = luteinizing hormone.

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EXPERIMENTAL

Animals

Male Sprague–Dawley rats (Möllergaards avlslaboratorier, Ejby, Denmark) were raised on a standard laboratory diet and kept on a 12 h light/12 dark cycle.

Chemicals

1α,25-Dihydroxy [26,27-methyl-^{3}H]cholecalciferol ([^{3}H]calcitriol) [160 Ci/mmol] was purchased from New England Nuclear. Unlabelled vitamin D_3 analogues (calcitriol and calcidiol) were kindly provided by Dr W. Meier, Roche, Basel. Bovine serum albumin (BSA), sodium molybdate (Na_2MoO_4), Triton-X-100, EDTA and Trizma base were obtained from Sigma Chemical Company. Hydroxylapatite (Bio-Gel HTP) was obtained from Bio-Rad; dithiothreitol (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 and [^{14}C]labelled ovalbumin and carbonic anhydrase were from New England Nuclear.

Cytosol preparation

Whole testis cytosol was prepared from decapsulated testes which were rinsed in ice-cold phosphate-buffered saline (PBS). The testes were then homogenized at 0°C in 5 vol of 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) in a Dounce all glass homogenizer (Kontes Glass Co., Vineland, NJ, U.S.A.) by 10 strokes with pestle A (loose-fitting) and 10 strokes with pestle B (tight-fitting). The homogenates 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. The protein content of each cytosol was measured by the method of Lowry *et al.*[5] using BSA as standard.

Binding assays

Cytosol from pools of testicular tissue was incubated with [^{3}H]calcitriol with and without an excess of unlabelled calcitriol at 0°C for the times indicated in the figure legends. Bound and free steroids were separated by hydroxylapatite assay or sucrose gradient centrifugation.

Hydroxylapatite assay

The hydroxylapatite assay was performed essentially as described by Wecksler and Norman[6]. In brief, a 50% slurry (v/v) of hydroxylapatite was prepared in KTEDMo. Labelled cytosol (100 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 3 times with 1.5 ml KTEDMo containing 0.5 Triton-X-100 by centrifugation at 2000 *g* for 5 min. The final hydroxylapatite pellet was extracted twice with 1.0 ml ethanol 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

Linear 5–20% (w/v) sucrose density gradients (4.2 ml) were prepared with a Buchler gradient mixer. Labelled cytosol (200 μl) was incubated with 50 μl DCC (dextran-coated charcoal; 2.5% charcoal, 0.25% 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 layered on top of the gradient and 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. After the centrifugation, fractions of 8 drops each were collected from the bottom and counted for radioactivity. BSA (4.4S), ovalbumin (3.7S) and carbonic anhydrase (3.0S) were used as external sedimentation marker proteins.

Differential displacement of [^{3}H]calcitriol binding to the serum DBP

In order to eliminate binding of [^{3}H]calcitriol to the serum-derived vitamin D binding protein (DBP), we utilized the following procedure: First, cytosol was labelled to equilibrium with [^{3}H]calcitriol (0.3–0.5 nM) with and without excess unlabelled calcitriol as described above. After labelling, an excess of unlabelled calcidiol (1.0 μM) was added, and the incubation was continued for 1 h at 0°C. Due to the rapid dissociation of [^{3}H]calcitriol from the DBP ($t_{1/2} < 5$ min) and the very slow dissociation from the receptor ($t_{1/2} \gg 48$ h), this procedure completely eliminated binding of [^{3}H]calcitriol to the DBP without reducing the binding to the receptor [3]. After the 1 h incubation with unlabelled calcidiol, bound and free steroids were separated by hydroxylapatite assay or sucrose gradient centrifugation as described above.

RESULTS AND DISCUSSION

Developmental changes in calcitriol receptors in testis cytosol

Figure 1 shows specific binding of [^{3}H]calcitriol in high-salt cytosol from whole testis of rats of various ages (19–272 days of age), using the hydroxylapatite batch assay. Figure 2 shows sucrose gradient centri-

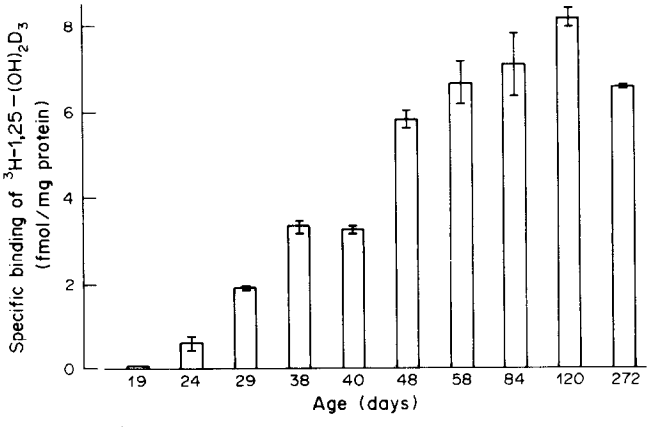

Fig. 1. Specific binding of [^{3}H]calcitriol (^{3}H-1,25-(OH)$_2$D$_3$) in whole testis cytosol at different ages. High-salt cytosol from whole testis of rats at the indicated ages were incubated at 0°C for 15 h with 0.4 nM [^{3}H]calcitriol with and without a 200-fold excess of unlabelled calcitriol. After labelling, the incubations were continued for 1 h at 0°C in the presence of 1 μM unlabelled calcidiol before estimation of specific binding with the hydroxylapatite assay. Mean ± SD of triplicate estimates.

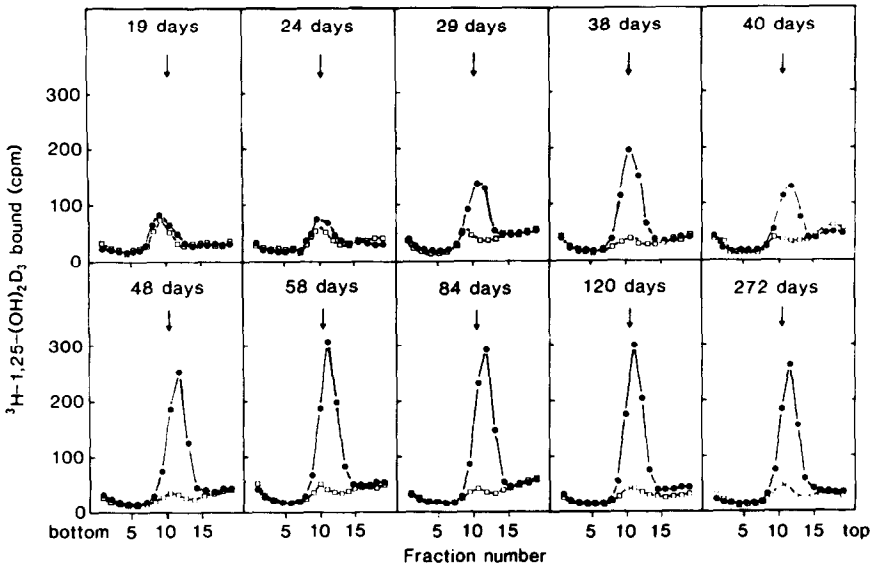

Fig. 2. Sucrose gradient analysis of [^{3}H]calcitriol (^{3}H-1,25-(OH)$_2$D$_3$) binding in whole testis cytosol at different ages. High-salt cytosols from whole whole testis of rats at the indicated ages were incubated at 0°C for 15 h with 0.4 nM [^{3}H]calcitriol with (□——□) and without (●——●) a 200-fold excess of unlabelled calcitriol. After labelling, the incubations were continued for 1 h at 0°C in the presence of 1 μM unlabelled calcidiol before charcoal adsorption of free steroids and sucrose gradient centrifugation as described under Experimental. The arrow denotes the ovalbumin external sedimentation marker at 3.7 S.

fugation of the same cytosol fractions. In both cases, binding of [^{3}H]calcitriol to the serum vitamin D binding protein (DBP) was removed by "differential displacement". As shown from the figures, little or no specific binding to receptors was seen in the cytosols from 19-day old rats. At 24 days of age, low but significant specific binding binding of [^{3}H]calcitriol was observed (Fig. 1). With increasing age, there was a gradual increase in specific binding of [^{3}H]calcitriol to the testicular receptors, reaching adult levels (6–8 fmol/mg protein) at 50–60 days of age.

In previous studies, in which high-salt cytosols from whole testis of immature and adult rats were incubated with [^{3}H]calcitriol in the presence and absence of an excess unlabelled calcitriol, the majority of the binding was to a protein sedimenting in the 5–6 S region of the sucrose gradients, and which displayed the properties of the serum vitamin D binding protein (DBP) [3, 7–9]. However, taking advantage of the greatly different rates of dissociation of [^{3}H]calcitriol from the receptor ($t_{1/2} > 48$ h) and from the DBP ($t_{1/2} < 5$ min) [3], we were able to measure receptor binding by a "differential displacement" technique. In these studies, high-salt cytosols from whole testes of rats at different ages were first labelled with [^{3}H]calcitriol until equilibrium (15–18 h), after which radioactivity bound to the plasma DBP was displaced by the addition of a large excess (1 μM) of unlabelled calcidiol. Under these circumstances, binding to the DBP could be completely eliminated [3].

It has previously been shown that germ cells from adult rat testis do not contain steroid receptors for androgens or estrogens [10]. In a previous paper, we were not able to demonstrate receptors for calcitriol in testicular germ cells from 32-day old rats [3]. Thus, assuming that the receptors for calcitriol are present only in the somatic cells of the testis, the increase in concentration in the cells containing these receptors is actually far greater than is shown by expressing specific binding per mg cytosol protein. In Table 1, the specific binding of [^{3}H]calcitriol in testis cytosols is calculated both per mg cytosol protein and per testis. As seen from this table, the number of receptors per testis increased more than 10-fold from day 29 until day 120 of age. At higher ages, there was no further increase in calcitriol receptors (Table 1).

Developmental changes in specific [^{3}H]calcitriol binding is not due to differences in receptor degradation or binding affinity

To eliminate the possibility that the apparent lack of receptors for calcitriol in high-salt cytosols from the immature rat testis could be due to degrading enzymes present in the immature testis, equal volumes of homogenates from immature and adult testis were combined before cytosol preparation. If factors causing receptor degradation were present in the immature tissue during homogenization, these should also destroy receptors present in testis homogenates from adult rats. In contrast, if such degradation of receptors does not occur, specific binding in the cytosol from the mixed homogenates should be exactly half of that found in the "adult control". Results from such experiments are shown in Table 2. Here, testis tissue from 23- and 131-day old rats was washed in PBS and homogenized in 5 vol KTEDMo

Table 1. Concentration of cytosolic calcitriol receptor in rat testis at different ages

Age (days)	Testis weight (g)	Hydroxylapatite fmol/mg protein	Hydroxylapatite fmol/testis	Sucrose gradients fmol/mg protein	Sucrose gradients fmol/testis
19	0.066	<0.4	<1.0	<0.4	<1.0
24	0.19	0.6	3.8	0.4	2.3
29	0.46	1.9	29	1.9	23
38	0.77	3.3	83	3.7	91
40	0.87	3.2	74	2.6	59
48	1.28	5.8	199	5.3	182
58	1.65	6.6	306	6.3	292
84	1.72	7.1	355	6.4	323
120	1.97	8.1	425	7.0	368
272	1.94	6.5	366	5.8	323

High-salt cytosols from whole testes were incubated as described in the legends to Figs 1 and 2, and specific binding was estimated by hydroxylapatite assay (mean of triplicate estimates) and sucrose gradient analysis (mean of duplicate estimates), respectively.

as described under Experimental. Equal aliquots of each homogenate were then combined and the mixture was left at 0°C for 45 min together with the separate homogenates to extract nuclear receptors. Cytosols were prepared from all three homogenates and incubated at 0°C for 24 h with 0.5 nM [^{3}H]calcitriol with and without a 200-fold excess of unlabelled calcitriol. After labelling, the incubations were continued for 1 h at 0°C in the presence of 1 μM unlabelled calcidiol before estimation of specific binding. Receptors for calcitriol were undetectable in the immature (23-day old) testis cytosol. Using hydroxylapatite assay and sucrose gradient centrifugation, the cytosol from the combined homogenates had a receptor concentration of 50 and 54%, respectively, compared to the "adult control". This shows that the calcitriol receptors originally present in the adult testis homogenate are not destroyed by factors present in the immature testis homogenate.

To ascertain that the lower specific binding of [^{3}H]calcitriol observed in the immature cytosols by single-point measurements (Figs 1 and 2) was not due to a change in the binding affinity of the receptors, we estimated the binding affinity and capacity of the calcitriol receptors in testis cytosols from 33- and 145-day old rats, using Scatchard analysis [11] (Fig. 3). As seen from the figure, the concentration of binding sites in the adult testis cytosol is approx 3-fold higher than that in the 33-day old testis cytosol (7.1 vs 2.3 fmol/mg protein). Furthermore, the apparent equilibrium dissociation constants (K_d) of the receptors in the two cytosols are very similar (1.1×10^{-11} M and 0.8×10^{-11} M in 145-day old and 33-day old, respectively). Thus, the increase in specific binding of [^{3}H]calcitriol during development represents an increase in the number of binding sites and does not reflect a change in binding affinity.

In a previous study [3], we characterized the receptor for calcitriol in the adult testis and found that the receptor was present in comparable concentrations (fmol/mg protein) in isolated seminiferous tubules and dissected interstitial tissue. In a further attempt to localize the receptor to specific cells (Sertoli cells, peritubular cells and germ cells), we were not able to show specific binding of [^{3}H]calcitriol to any of these cells [3]. The fact that the isolation of Sertoli cells and peritubular cells were done on testes from immature (19-day old) rats, indicate that our previous inability to demonstrate calcitriol receptors in isolated cultured cells may be due to the age of the animals used rather than to the disappearance of calcitriol receptors during cell culture. It has, however, been shown that receptors for calcitriol in cells from other tissues may change during culture. In a study on cultured fetal rat calvaria, Manolagas *et al.*[12] demonstrated that calcitriol receptors disappeared during culture. To what extent receptors in testicular somatic cells may change during culture must await preparation procedures of isolated testicular cells from older animals.

Dramatic variation in the concentration of calcitriol receptors during development has also been reported in the rat intestinal mucosa. Halloran and DeLuca[13] showed that the receptor concentration

Table 2. Specific binding of [^{3}H]calcitriol in high salt testis cytosol from 23- and 131-day old rats, and in cytosol from combined testis homogenates (equal volumes) from rats at the two ages

Cytosol	Calcitriol receptor concentration (% of 131-day old) Hydroxylapatite	Sucrose gradients
23-Day old	ND	ND
131-Day old	100	100
Cytosol from combined homogenates	50 ± 4	54

ND = Not detectable.
Specific binding in adult cytosol (4.7 fmol/mg protein) is 100%.

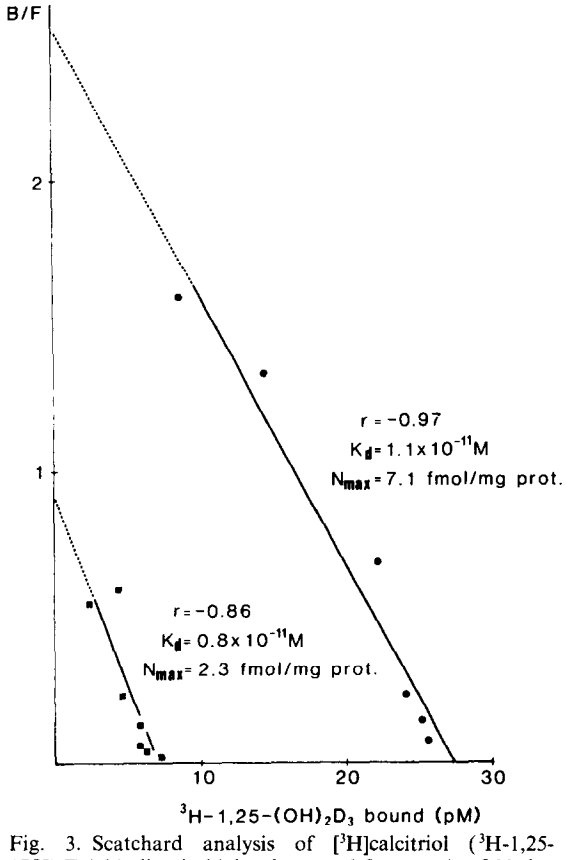

Fig. 3. Scatchard analysis of [^{3}H]calcitriol (^{3}H-1,25-(OH)$_2$D$_3$) binding in high salt cytosol from testis of 33-day old and 145-day old rats. Cytosols (33-day old: 3.3 mg protein/ml; 145-day old: 4.3 mg protein/ml) were incubated at 0°C for 49 h (33-day old) or 53 h (145-day old) with increasing concentrations of [^{3}H]calcitriol (0.01–0.4 nM) with and without a 200-fold excess of unlabelled calcitriol. One-hundred μl aliquots (triplicates) from each incubation tube were assayed for specific binding of [^{3}H]calcitriol using the hydroxylapatite assay. The figure shows Scatchard analysis of the specific binding; (■——■): 33-day old; (●——●): 145-day old.

was very low prior to day 14 post partum, after which the receptor levels increased steeply, reaching adult levels at day 28 of age. The appearance of calcitriol receptors in cells from the intestinal mucosa corresponded with the age at which responsiveness to the actions of calcitriol was aquired.

Walters *et al.*[1] recently demonstratred that estrogen-stimulated uterine growth was paralleled by an increase in uterine receptors for calcitriol. Examining receptor levels in testis, a doubling in receptor concentrations was seen between 37 and 90 days of age [1]. However, the levels of receptors reported in their study (5–10 fmol/g tissue) was approx 20 times lower than that observed in the present study (Table 1). In spite of that, both studies show a significant increase in receptors for calcitriol during pubertal development.

The possible stimulus for the rapid increase in calcitriol receptors in the testis after day 25 is unknown. An interesting speculation, which still has to be verified, is the possibility that this may be related to the dietary intake of calcium. In the present experiments, the receptors appeared shortly after weaning. However, to what extent earlier weaning may be associated with premature appearance of testicular calcitriol receptors remains to be established.

The lack of testicular calcitriol receptors before day 24 of age indicates that calcitriol may be of little importance for testicular function in immature rats. The role of calcitriol in the pubertal (25–50 days) and adult (> 50 days) testis is still not known. It has been claimed that calcitriol deficiency induced by nephrectomy in rats is associated with a decreased responsiveness of the Leydig cells to LH [14]. The possible role of calcitriol in the seminiferous tubules has not been examined, but it may be related to calcium homeostasis in Sertoli cells. It is interesting to note that the appearance of calcitriol receptors in the testis occurs shortly after the initiation of Sertoli cell secretory activity [15], and it is well known that calcium is of major importance for many secretory mechanisms [16].

In conclusion, in the present paper we have shown that the testicular receptor for calcitriol is undetectable in high-salt cytosols from immature rat testis. The receptors appear at day 24, and the levels increase rapidly during pubertal development, reaching adult levels around day 50–60. It is likely that the lack of calcitriol receptors in cultured testicular cells from immature (19-day old) rats [3] may primarily be due to the age of the animals used.

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