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CHARACTERISATION OF RECEPTORS FOR 1,25-DIHYDROXYVITAMIN D_3 IN THE HUMAN TESTIS

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Summary—Specific high affinity receptors for 1,25-dihydroxyvitamin D_3 have been demonstrated in the human testes. The mean binding affinity ($K_d \pm$ SD) of the receptor for 1,25-dihydroxyvitamin D_3 was $1.75 \pm 0.32 \times 10^{-10}$ M but the binding capacity was low (mean $N_{max} \pm$ SD = $0.53 \pm 0{,}18$ fmol/mg protein). Binding was time- and temperature-dependent, with a maximum binding achieved after 1 h at 25°C. Although binding also took place at 4 and 37°C, higher and more rapid binding was found at 25°C. Furthermore, the binding between the ligand and the receptor was specific since only unlabelled 1,25-dihydroxyvitamin D_3 competed with the labelled ligand. Binding of 1,25-dihydroxyvitamin D_3 was abolished by trypsin and heat. Sucrose density gradient centrifugation revealed a sedimentation coefficient of 3.6S.

INTRODUCTION

Vitamin D_3 is produced in the skin and carried in the blood stream to the liver where it is hydroxylated to from 25-hydroxyvitamin D_3. The final step in the formation of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) takes place in the kidney where 25-OH-D_3 is hydroxylated in the 1α position [1–4]. 1,25$(OH)_2D_3$ is an antirachitic agent and is a general regulator of calcium and phosphate metabolism, working mainly in bone, intestine and kidney. Other important roles for 1,25-$(OH)_2D_3$ include controlling secretion of polypeptide hormones [5], promoting induction of enzymes responsible for the catabolism of the hormone [3], controlling cellular proliferation and differentiation [6] and modulating certain immune system functions [7].

1,25-$(OH)_2D_3$ interacts with soluble intracellular receptors in its target tissues to alter mRNA transcription and protein synthesis [4, 8, 9]. The unoccupied receptors seem to be localised in chromatin [10] or nuclei [11]. The receptor has been localised in certain cell types including those of the intestinal mucosa [12]. Physical studies of the receptor revealed that in man the receptor from intestinal cytosol sediments at 3.5S and has a molecular weight of 60 kDa. Furthermore the affinity for the receptor molecule ranges from 10^{-10} to 5×10^{-11} M.

There are several factors which suggest that vitamin D_3 might play a role in fertility and spermatogenesis: firstly the presence of vitamin D_3 receptors in seminiferous tubules and interstitial tissues of adult rat testes [13]; secondly, the demonstration of calcium binding proteins in testes, though these have not been shown to be vitamin D-dependent [14,15] and the fact that in vitamin D-deprived animals a disturbance in reproduction was found resulting in a reduction of 75% in overall fertility [16]; thirdly, *in vivo* administration of 1,25-$(OH)_2D_3$ completely restores the nephrectomy mediated reduction in LH stimulated cAMP formation in the testis [17]. However, since no one has reported the presence of receptors for 1,25-$(OH)_2D_3$ in human testes, we have undertaken the present study in order to characterise 1,25-$(OH)_2D_3$ receptors in human testicular tissue.

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EXPERIMENTAL

Materials

[^{3}H]1,25-$(OH)_2D_3$ (sp. act: 176 Ci/mmol) was obtained from Amersham International, Bucks. Non-radioactive 24-OHD_3 and 1,25-$(OH)_2D_3$ were gifts from Roche Products, Welwyn Garden City, Herts. Other non radioactive steroids, marker proteins and hydroxylapatite were from Sigma, Poole.

The following buffers were used: TEDMO buffer containing 100 mM Tris/HCl, 1.5 mM EDTA, 1 mM dithiothreitol and 10 mM Sodium molybdate, pH 7.4; TEDMOK buffer which was 0.3 M KCl in TEDMO pH 7.4.

Tissues

Normal human testicular specimens were obtained from patients with prostate cancer who were undergoing bilateral orchidectomy. The specimens were immediately frozen in liquid nitrogen and stored at −70°C.

Methods

Tissue preparation. This was based on the method of Levy *et al.*[13] and was carried out at 0–4°C.

1 g testicular tissue was homogenised in 2 vol of TEDMOK buffer with a Ystral homogeniser (Scientific Instrument Centre Ltd, Liverpool). The resultant homogenate was filtered through one layer of nylon gauze (Nybolt 9–150, John Stainer & Co., Manchester), and centrifuged for 1 h at 100,000 *g* in a Sorval OTD-65 Ultracentrifuge to obtain the high salt supernatant (S_{100}) fraction. Protein was determined in this fraction by the method of Bradford [18].

Binding studies. 190 μl samples of S_{100} were incubated with 0.4 nM [^{3}H]1,25-$(OH)_2D_3$ for various times at 4, 25 and 37°C, in duplicate, in the presence and absence of 100-fold excess unlabelled 1,25-$(OH)_2D_3$. Separation of bound from free ligand was achieved by addition of 0.5 ml of a well washed slurry of hydroxylapatite in TEDMOK buffer to the incubation medium, with shakings for 20 min. The suspension was subsequently centrifuged for 5 min at 2000 *g*. The supernatant was discarded and the pellet washed in TEDMOK buffer and the process repeated four times. After the final wash, the hydroxylapatite was extracted with 2 ml ethanol at room temperature and the layers separated by centrifugation and counted.

For saturation studies, the S_{100} was incubated for 1 h at 25°C, in duplicate, with increasing concentrations (0–570 pM) of radiolabelled 1,25-$(OH)_2D_3$, in the presence and absence of 100-fold excess unlabelled 1,25-$(OH)_2D_3$. The 1,25-$(OH)_2D_3$ bound complex was separated from free hormone by the hydroxylapatite technique as described above. The dissociation constant (K_d) and the number of binding sites in the receptor were calculated by the Scatchard method [19].

Competition studies. Binding specificity was studied by incubating the cytosol with radiolabelled 1,25-$(OH)_2D_3$ with or without a 100-fold molar excess of unlabelled 1,25-$(OH)_2D_3$, testosterone, progesterone, oestrogen, cholecalciferol (vitamin D_3) or 25-$(OH)D_3$. After incubation, the same steps were followed as described under binding studies.

Heat and trypsin studies. S_{100} was either pretreated with trypsin (1 mg/mg protein) for 1 h at 25°C or preheated at 45°C for 15 min. After the preincubation, [^{3}H]1,25-$(OH)_2D_3$ was added with and without 100-fold excess of unlabelled 1,25-$(OH)_2D_3$ and the incubation was continued for 1 h at 25°C before estimation of specific binding by the hydroxylapatite assay.

Sucrose density gradient centrifugation. Linear sucrose density gradients (5–30%; w/v) were prepared by layering 0.84 ml aliquots of 5, 10, 15, 20, 25 and 30% sucrose solution in TEDMO (low salt) buffer in cellulose nitrate centrifugation tubes. After equilibration at room temperature overnight, 200 μl of 1,25-$(OH)_2D_3$ bound complex extracted in TEDMO buffer was layered on the gradient. 200 μl of bovine serum albumin (BSA), ovalbumin and myoglobin with sedimentation coefficients of 4.0, 3.6 and 3.0S respectively were run on separate parallel gradients as external standards. The tubes were centrifuged at 60,000 rpm for 2 h at 4°C in a Sorval TV 865 vertical rotor (DuPont Instruments). Fractions of 6 drops were harvested into each of 20 test tubes, by upward

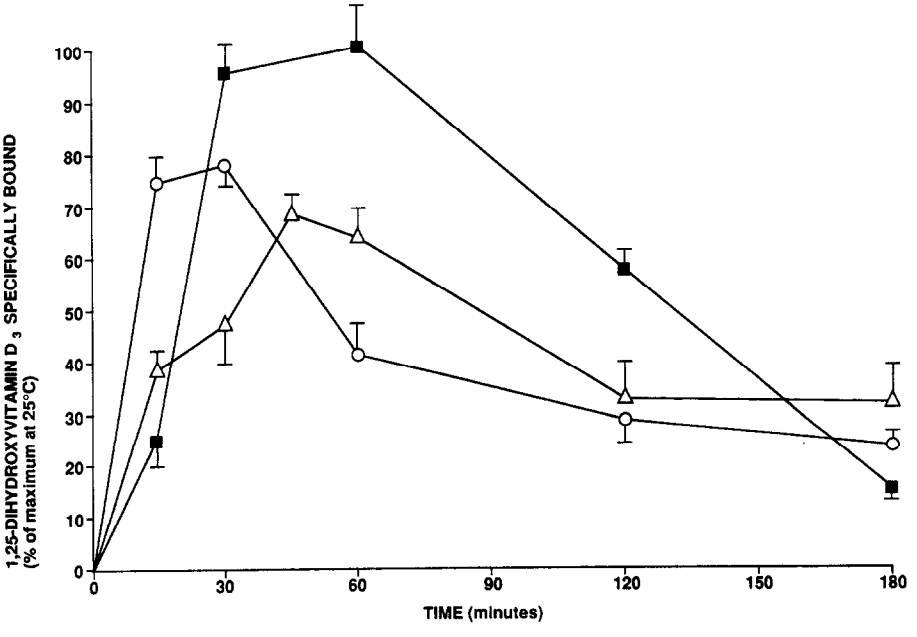

Fig. 1. The effect of time and temperature on specific binding. Testicular S_{100} (190 μl) was incubated at 4°C (○——○); 25°C (■——■); and 37°C (△——△) with 400 pM [^{3}H]1,25-$(OH)_2D_3$, in the presence and absence of 100-fold excess cold ligand for up to 180 min. The specific binding was determined as described in "Experimental" using the hydroxylapatite assay. Each value represents the mean ± SD of 3 different experiments.

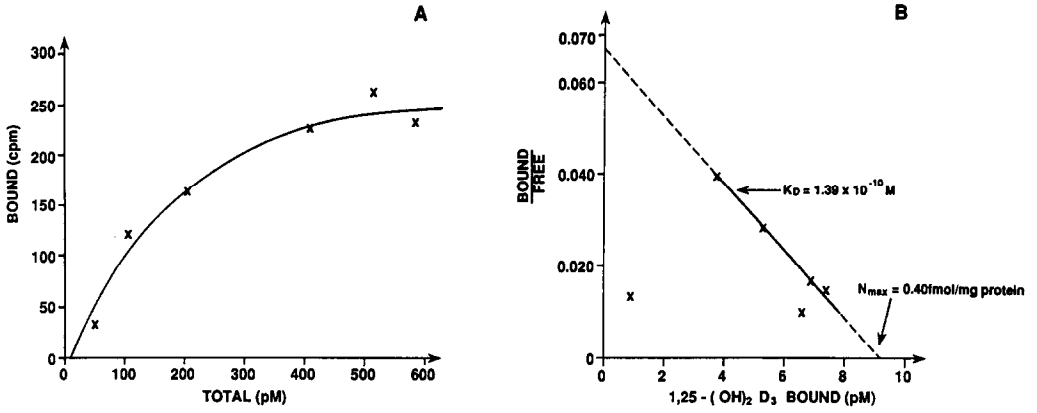

Fig. 2. Scatchard analysis of [^{3}H]1,25-$(OH)_2D_3$ in human testicular cytosol Increasing concentrations of labelled 1,25-$(OH)_2D_3$ (0–0.57 nM) were incubated with 190 μl S_{100} in the presence and absence of 100-fold excess unlabelled 1,25-$(OH)_2D_3$ for 1 h at 25°C. Specific binding obtained at each concentration was calculated and plotted (A). The data obtained from the saturation curves was used to calculate the dissociation constant and the number of binding sites by the Scatchard method (B).

displacement using 40% sucrose. The harvested fractions were counted in 6 ml of scintillation cocktail.

RESULTS

Testicular cytosol (190 μl) was incubated at 4, 25 and 37°C for up to 180 min with 0.4 nM [^{3}H]1,25-$(OH)_2D_3$. Maximal binding was obtained at 25°C (Fig. 1). At this temperature, binding increased with time until 60 min after which it decreased until 180 min. Although similar patterns were obtained at 4 and 37°C, the binding at these temperatures was lower. All subsequent incubations were therefore performed at 25°C for 1 h.

Increasing concentrations of labelled 1,25-$(OH)_2D_3$ (0–0.57 nM) were incubated with cytosol in the presence or absence of 100-fold excess of unlabelled 1,25-$(OH)_2D_3$. Figure 2A shows that saturation of the receptors occurs at a concentration of about 0.5 nM. Data obtained from the saturation studies on these tissues were used to calculated the dissociation constant (K_d) and the number of binding sites. Scatchard plot analysis was consistent with a straight line indicating a single class of binding sites ($K_d \pm$ SD $= 1.75 \pm 0.32 \times 10^{-10}$ M; $n = 3$). The number of binding sites (mean $\pm$ SD) $= 0.53 \pm 0.18$ fmol/mg protein. A typical Scatchard plot is shown in Fig. 2B.

No other unlabelled steroid or vitamin D_3 metabolite could compete with labelled 1,25-$(OH)_2D_3$ apart from unlabelled 1,25-$(OH)_2D_3$ which showed a 100% competition (Fig. 3). Heat and trypsin pretreatment (Fig. 4) abolished the specific binding indicating that 1,25-$(OH)_2D_3$ binds to a soluble protein.

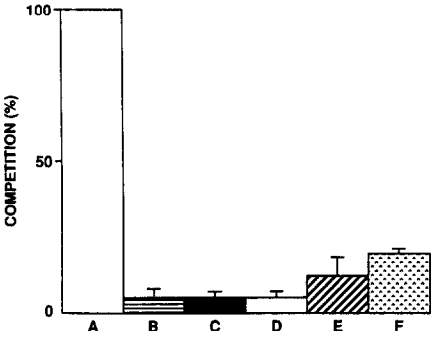

Fig. 3. Specificity of 1,25-$(OH)_2D_3$ binding sites. Specificity was studied by incubating 190 μl testicular S_{100} with radiolabelled 1,25-$(OH)_2D_3$ (500 pM) for 1 h at 25°C with or without a 100-fold molar excess of unlabelled: 1,25-$(OH)_2D_3$ (A), testosterone (B), oesterogen (C), progesterone (D), vitamin D_3 (E) and 25-$(OH)D_3$ (F). 100% competition was taken as the amount of [^{3}H]1,25-$(OH)_2D_3$ displayed by unlabelled 1,25-$(OH)_2D_3$. Each value was determined in triplicate and values are depicted as means $\pm$ SD.

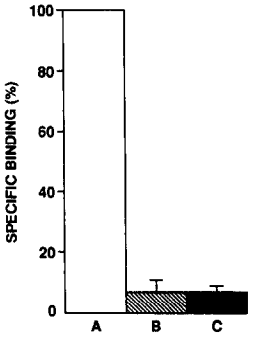

Fig. 4. Inactivation of [^{3}H]1,25-$(OH)_2D_3$ binding by heat and trypsin treatments. S_{100} was treated with either 0.05% trypsin for 1 h or heated to 60°C for 15 min. Thereafter, portions (190 μl) of the treated samples were incubated with 0.5 nM of [^{3}H]1,25-$(OH)_2D_3$ for 1 h at 25°C in the presence and absence of 100-fold excess unlabelled 1,25-$(OH)_2D_3$. Specific binding was calculated as described in "Experimental". Untreated samples repesented 100% specific binding. Studies were performed in triplicate and were: untreated (A), trypsin (B), and heat (C). Values represented the mean $\pm$ SD.

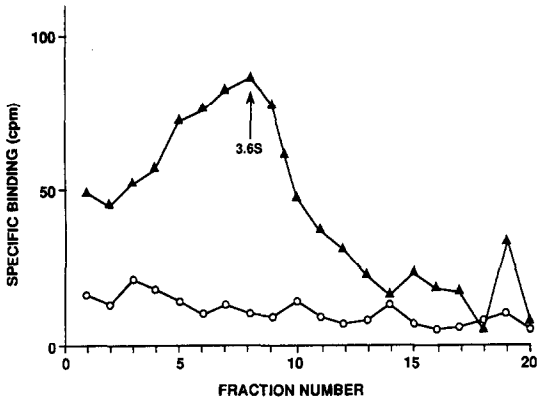

Fig. 5. Linear sucrose density gradients (5–30%) prepared in TEDMO (low salt) buffer pH 7.4 were equilibriated at room temperature overnight. Samples of bound 1,25-$(OH)_2D_3$ receptor complex were layered on the gradients and were centrifuged at 60,000 rpm for 2 h. Myoglobin (3.6S), albumin (4.6S) and ovalbumin (2.0S) served as standards (data not shown). Samples were collected by upward displacement using 40% sucrose solution and counted in the scintillation counter. [^{3}H]1,25-$(OH)_2D_3$ and cytosol at only (△——△); [^{3}H]1,25-$(OH)_2D_3$ and cytosol in the presence of 100-fold excess unlabelled 1,25-$(OH)_2D_3$ (○——○).

Figure 5 shows a peak of binding with a sedimentation coefficient of 3.6S. The peak was abolished in the presence of 100-fold excess of cold 1,25-$(OH)_2D_3$. In contrast, testosterone, progesterone, oestrogen, cholecalciferol and 25-(OH)D_3 had no effect on the peak (results not shown).

DISCUSSION

The present study shows the presence of a binding protein for 1,25-$(OH)_2D_3$ in high salt extracts from human testis with properties (affinity, specificity, sedimentation coefficient and binding kinetics) similar to the receptors for 1,25-$(OH)_2D_3$ described in other tissues [1, 2, 4, 9, 20, 21]. Maximum specific binding was obtained after incubation for 1 h at 25°C (Fig. 1), in agreement with previous work on fish [21], human breast tissue [22] and chick intestinal mucosa [23]. These workers observed a maximum binding after incubating at 25°C for 2 h, a period which yielded minimum binding in the present study. Rat testis extracts, however, produced their maximum binding after incubation at 4°C overnight, suggesting species differences. Scatchard plot analysis revealed high affinity receptor proteins ($K_d \pm$ SD = $1.75 \pm 0.32 \times 10^{-10}$ M; $n = 3$). This is in agreement with other values ($1.4–2.6 \times 10^{-10}$ M) for the rat testis [14] and human intestinal receptors [22] but at variance with that of Levy *et al.*[13] who quoted 3×10^{-11} M for rat testicular receptor.

The receptor concentrations obtained in this study ($N_{max} = 0.53 \pm 0.18$ fmol/mg cytosol protein) are close to that found by Walters *et al.*[15] for the rat testis. These findings indicate that the receptor levels in the testis of rat and human are relatively lower than those measured in other target tissues. The reason for these low levels is not clear, but may be related to differences in extraction. The apparent subcellular distribution of receptors is influenced by the salt concentration of the extraction buffers employed [10]: high ionic strength buffers, such as those used in the present study, favour an accumulation of receptor in the S_{100} fraction whereas low salts lead to their retention in the nuclear fraction. However, there is also evidence to suggest that a substantial amount of chromatin bound 1,25-$(OH)_2D_3$ is resistant to high salt extraction [9].

Competition studies demonstrated the specificity of the binding of 1,25-$(OH)_2D_3$ since no other unlabelled steroid tested apart from 1,25-$(OH)_2D_3$ (Fig. 3) displayed the ligand from the receptor; this is in agreement with earlier studies [24]. The effect of heat and trypsin pretreatment (Fig. 4) indicates that the receptor is a proteinaceous macromolecule associated with testicular tissue. A sucrose density gradient experiment revealed a sedimentation coefficient of 3.6S for the 1,25-$(OH)_2D_3$ receptor in agreement with values reported by others [21, 25].

This study shows for the first time that the human testis contains a binding protein with identical characteristics to those described for the receptor for 1,25-$(OH)_2D_3$. The significance of this finding is at the moment unclear, but there are reports implicating 1,25-$(OH)_2D_3$ receptors in spermatogenesis and fertility. However, the earlier experiments have been confined to animal models [13–17] and clearly more work will have to be done on human tissue prior to ascribing a specific role for vitamin D_3 in man.

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