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PHYSICAL SEPARATION OF AORTIC CORTICOID RECEPTORS WITH TYPE I AND TYPE II SPECIFICITIES

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Summary—Previous gel filtration binding assay studies indicated that rat vascular smooth muscle cells contained corticoid receptor I and corticoid receptor II sites which could be distinguished on the basis of their relative affinities for aldosterone and dexamethasone. Ion-exchange chromatography experiments were designed to separate the two sites for further studies on their physical characteristics and role in vascular smooth muscle cell physiology. Cultured aortic cells were incubated with 5-10 nM ^{3}H steroid alone or in the presence of 10-fold non-radioactive steroid competitor for 30 min at 37°C. Following cell lysis, total cellular protein-bound steroid was isolated using Sephadex G-25 and applied to a DEAE-cellulose ion-exchange column. Three peaks of radioactivity were eluted using a 1–200 mM sodium phosphate gradient: peak I (30–38 mM), peak II (52–64 mM), and peak III (92–102 mM). Peaks I and II contained 60% of the eluted radioactivity and exhibited the same steroid specificity as corticoid receptor II sites (dexamethasone > aldosterone). Peak III contained 40% of the eluted radioactivity and exhibited the same steroid specificity as corticoid receptor I sites (aldosterone > dexamethasone). These studies support the binding assay data on steroid specificity and relative proportion of type I and II sites. They also document the existence of type I and II corticoid receptors with different physicochemical characteristics in rat aortic smooth muscle cells.

INTRODUCTION

By the mid-seventies, corticosteroid receptors had been discovered in a multitude of glucocorticoid and mineralocorticoid target tissues [1]. Two classes of receptors were described: type I mineralocorticoid receptors with a binding hierarchy of Aldo > B > DM and type II glucocorticoid receptors with DM > B > Aldo. Since then, additional corticosteroid binding sites have been reported in a variety of classical and non-classical targets [2]. These include type I, type II and B-preferring sites with a binding hierarchy of B = Aldo > DM. Recent studies show that the intrinsic specificities of type I receptors in kidney and B-preferring sites in the hippocampus are identical and suggest that the occupancy of these sites by Aldo in mineralocorticoid targets is determined by the sequestration of B by tissue transcortin [3, 4].

Vascular smooth muscle cells cultured from rat aorta also contain both type I sites with B-preferring specificity and type II receptors [5, 6]. Steroid effects on protein synthesis have been described in these cells and specificity studies indicate they are mediated by type II glucocorticoid receptors and not type I sites [7]. Recently, Moura and Worcel[8] have demonstrated in arterial smooth muscle, mineralocorticoid-specific effects on Na transport that are consistent with a type I receptor-initiated, mRNA-mediated mechanism of steroid action. The latter report is especially intriguing, since we have found a rat model of resistance to mineralocorticoid and salt hypertension with lower affinity aortic corticoid I receptors compared to a salt-sensitive model [9].

Paramount to further characterization and comparison of aortic corticoid receptors is the physical separation of binding sites with mineralocorticoid (type I) and glucocorticoid (type II) specificities. We have used ion-exchange chromatography (IEC), a technique of high resolving power for steroid receptors [10–12], to examine the heterogeneity of aortic corticoid binding sites. In this paper we provide evidence for two corticoid receptors with different physical characteristics in rat vascular smooth muscle cells.

EXPERIMENTAL

Reagents

Radioactive steroids were purchased from New England Nuclear: [^{3}H]Aldo, 80–105 Ci/mmol, [^{3}H]B, 80–105 Ci/mmol, and [^{3}H]DM, 35–50 Ci/mmol. Non-radioactive steroids were purchased from Sigma, Steraloids, and Calbiochem-Behring. Steroids were stored as stock solutions in absolute ethanol at 4°C

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The following trivial names and abbreviations are used: RU26988 = 11β,17β-dihydroxy-21-methyl-17α-pregna-1,4,6-trien-20-yn-3-one; dexamethasone (DM), 9α-fluoro-11β,17,21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione; aldosterone (Aldo); corticosterone (B); deoxycorticosterone (DOC).

and checked for purity by thin layer chromatography. Tissue culture materials were obtained from Grand Island Biological Company. Sephadex G-25 was obtained from Pharmacia and DEAE-cellulose DE-52 was a product of Whatman Biochemicals.

Cell cultures

Male Wistar–Kyoto rats, age 12–14 weeks, were obtained from Charles River. Vascular smooth muscle cells were cultured from aortic explants by conventional tissue culture techniques as described previously [5, 6]. By morphological and immunohistochemical identification methods, the established cultures contained exclusively smooth muscle cells which had modulated phenotypically in culture from a contractile to a proliferative state [13].

In addition, these cultures do not metabolize Aldo and B during a 30 min incubation or subsequent gel filtration binding assay, as determined by extraction and thin layer chromatography [5].

Ion-exchange chromatography

The procedure of Agarwal[14] for IEC of renal mineralocorticoid receptors was adapted for use in chromatographic demonstration and comparison of aortic corticoid receptors. IEC was performed on DEAE-cellulose (DE-52) columns equilibrated with 60–70 ml of 1 mM Na-phosphate buffer (pH 7.5) containing 20% glycerol, at a flow rate of 35 ml/h. All manipulations were carried out at 4°C in a cold room. The resin bed dimensions were 0.9 cm × 25 cm. Pharmacia K9/30 acrylic plastic columns were packed by gravity-induced flow and the resin bed shrank 2 cm during equilibration.

Quadruplicate cultures (approx. 15×10^6 cells) from each aortic cell line were incubated with ^{3}H-steroid plus or minus non-radioactive steroid for 30 min at 37°C. The cells were harvested by trypsin treatment and suspended in a solution containing 0.02 M Tris–HCl (pH 7.5), 0.32 M sucrose, and 1 mg/ml bovine gammaglobulin at 4°C. From this point on all experimental procedures were done at 4°C. After centrifugation for 20 min at 1600 *g*, the cells were resuspended in 1 ml of 10 mM Na-phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.5 M KCl, and 20% glycerol (starting buffer) and sonicated (Branson water bath sonicator) to break up the cell and nuclear membranes. After centrifugation for 10 min at 2800 *g* to pellet cell debris and membranes, an aliquot of the supernatant was saved for assay of DNA concentration [15] and the remainder was desalted by passage through Sephadex G-25 columns equilibrated with 1 mM Na-phosphate buffer (pH 7.5) containing 20% glycerol. This procedure also removed free steroid from the sample.

The void volume peak of protein-bound radioactivity (approx. 5 ml) was manually applied to the top of the DE-52 resin bed with a Pasteur pipette. Subsequently, the column was washed with 60–70 ml of the starting buffer (pre-wash) and 4 ml fractions were collected. The protein was eluted with a 120 ml linear gradient of 1 mM–200 mM Na-phosphate (pH 7.5) in 20% glycerol containing 1 mM EDTA at a flow rate of 35 ml/h (fraction volume = 2 ml). The radioactivity of the pre-wash and elution fraction was determined by liquid scintillation counting and corrected for background and efficiency. Recoveries were based on the radioactivity (dpm) of the applied sample (G-25 void volume peak of protein-bound steroid) and were 65–70% for whole cell receptor preparations. Conductivity was measured with a Chemtrix Type 70 conductivity meter. Protein was detected by absorbance at 280 nm in a Zeiss PMQII spectrophotometer. Elution profiles were graphed using the CLINFO Data Management and Analysis System (supported by the Clinical Research Center Program Grant No. RR73, The University of Texas Medical Branch, Galveston) and peaks were quantitated by geometrical estimation of area under the curve. In all the examples shown, the samples contained approx. 300 μg DNA.

Under these conditions, free steroid did not bind to the anion exchange column. All attempts to elute additional protein-bound steroid following gradient elution with 1–200 mM Na-phosphate by step-wise elution with up to 1 M NaCl were unsuccessful. Separation of the eluted peaks was improved by the inclusion of 1 mM EDTA in the gradient buffers. Recoveries could be improved (90–95%) by decreasing the resin bed volume, however, the resolution between eluted peaks was markedly decreased, even in the presence of EDTA.

Sources of error

At least three sources of error in IEC elution profiles of vascular steroid binding were apparent. First, following removal of free steroid by G-25 filtration chromatography, only 65–70% of the applied counts were subsequently recovered from the anion-exchange column. Since decreasing the resin bed volume increased the recovery to 90–95%, lower recoveries can be explained by non-specific retention of protein bound steroid. Secondly, the pre-wash peak contained a large amount of the recovered radioactivity. Since free steroid is not retained by the column, the pre-wash peak may be heterogeneous and therefore, difficult to assess qualitatively and quantitatively. Another source of error was due to receptor instability and was introduced when time, temperature or sonication varied between experiments and could result in qualitative and quantitative variation in the recovered peaks.

RESULTS

Resolution of steroid-binding peaks by IEC

Rat aortic smooth muscle cells were incubated with 2–10 nM [^{3}H]Aldo at 37°C and 90% of the specific-binding as well as non-specific binding was

extracted by sonicating cells in a buffer containing 0.5 M KCl. This material was desalted by Sephadex G-25 gel filtration which also removes free steroid. The void volume G-25 peak was then applied to a long DE-52 ion-exchange column and a representative elution profile is shown in Fig. 1A. Four peaks are apparent; the first peak is the pre-wash peak which contains radioactivity not retained by the resin and eluted with the starting buffer and three peaks (I, II and III), that were eluted with increasing phosphate concentration. When the same experiment was performed in the presence of a 500-fold excess of non-radioactive Aldo, all four peaks were displaced (Fig. 1B). Under these conditions, the radioactivity remaining in the pre-wash peak could represent non-specific binding or dissociated steroid.

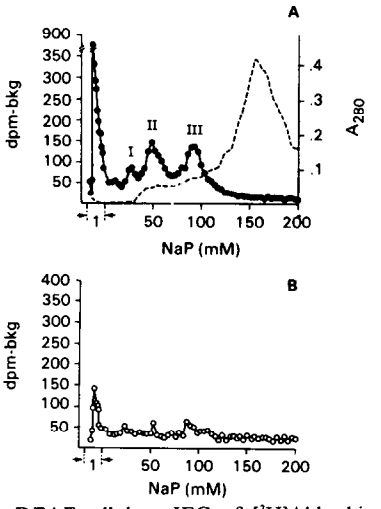

Fig. 1. DEAE-cellulose IEC of [^{3}H]Aldo binding in rat aortic smooth muscle cells. A. Aortic cells were incubated with 5 nM [^{3}H]Aldo at 37°C for 30 min. Whole cell sonicates were freshly prepared in starting buffer and desalted by passage through a Sephadex G-25 column before loading into the pre-equilibrated anion exchange resin. The first peak represents radioactivity not retained during a 60 ml pre-wash with starting buffer (arrows, 1 mM Na-phosphate). Fractions were eluted with a 1–200 mM Na-phosphate gradient and counted for determination of radioactivity (closed circles), which was distributed in three peaks, I, II and III. Linearity of the Na-phosphate gradient was established by conductivity measurements, and fraction number was expressed as mM Na-phosphate. Protein was measured by absorbance at 280 nm (dashed line). B. Steroid–receptor complexes were prepared and IEC conducted in the same manner as A., except cells were incubated with 5 nM [^{3}H]Aldo in the presence of 500-fold excess nonradioactive Aldo.

Steroid specificity of peaks resolved by IEC

A series of IEC experiments with cells incubated with various [^{3}H]steroids in the presence or absence of 10-fold competing non-radioactive steroids determined the steroid specificity of the peaks resolved in Fig. 1. The results for all steroids are summarized in Table 1 and the elution profiles for 10 nm [^{3}H]B alone and in the presence of 100 nM Aldo and DM are shown in Fig. 2 (A–C). The variability in elution molarities of the peaks between experiments is shown in Table 1 and represents a variation of less than two fractions.

Peaks I and II are glucocorticoid-specific, since they are labelled with all steroids and displaced by DM and B but not Aldo. Peak III is labelled by Aldo and B but not DM and is displaced by Aldo and B but not DM. When [^{3}H]DOC was used as the ligand, a heterogeneous pattern resulted which was always characterized by a broad peak of radioactivity near the end of the elution profile which was not displaced by 10-fold unlabelled DOC (not shown). The pre-wash peak was labelled and reduced by all steroids.

Correlation between IEC and binding assay data

Previous studies demonstrated binding of [^{3}H]Aldo in cultures of rat aortic smooth muscle cells [5]. Scatchard analysis of these data resulted in curvi-

Table 1. Steroid specificity of peaks resolved by IEC

Incubation conditions	*N*	Na-phosphate elution molarity (mM) (mean ± SD) Peak I 34 ± 4	Peak II 58 ± 6	Peak III 97 ± 5
5–10 nM [^{3}H]Aldo	6	+	+	+
5–10 nM [^{3}H]Aldo + 10-fold DM	2	–	–	–
5–10 nM [^{3}H]Aldo + 10-fold B	2	–	–	–
10 nM [^{3}H]B	2	+	+	+
10 nM [^{3}H]B + 10-fold Aldo	3	+	+	–/↓[a]
10 nM [^{3}H]B + 10-fold DM	2	–	–/↓[b]	+
2–10 nM [^{3}H]DM	4	+	+	–

N, No. of IEC experiments on different cell incubations.
+, present; –, missing, ↓, reduced.
[a]Peak III missing once, reduced twice.
[b]Peak II missing once, reduced once.

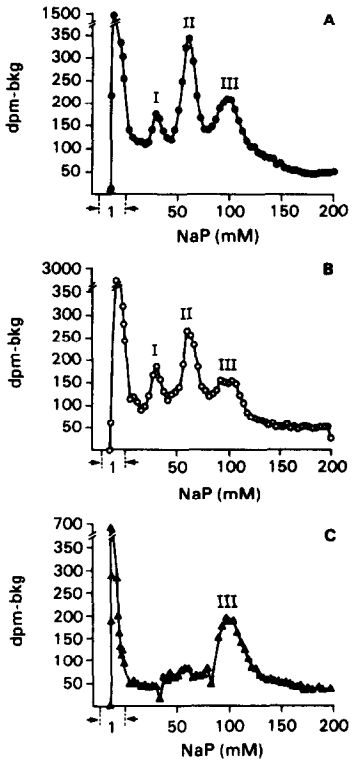

Fig. 2. IEC elution profiles showing steroid specificity of resolved peaks. Steroid–receptor complexes were prepared and IEC conducted in the same manner as that described in Fig. 1. A. When cells were incubated with 10 nM [^{3}H]B, three peaks (I, II and III) were eluted at the same positions as Aldo–receptor complexes shown in Fig. 1A. B. In the presence of 10 nM [^{3}H]B plus 10-fold nonradioactive Aldo, the only significant change was 30% reduction in the area of peak III. C. In the presence of 10 nM [^{3}H]B plus 10-fold nonradioactive DM, peaks I and II were displaced but not peak III.

linear plots suggesting two classes of binding sites: corticoid receptor I with high affinity and low capacity and corticoid receptor II with lower affinity and higher capacity. When cells were incubated with 2 nM [^{3}H]Aldo in the presence of 40 nM DM, a linear Scatchard plot was obtained [6]. The binding statistics derived from this plot were characteristic of corticoid receptor I, indicating that binding to corticoid receptor II sites had been displaced by DM. A similar experiment on IEC is shown in Fig. 3. Peaks I and II were displaced by 40 nM DM and therefore correspond to corticoid receptor II sites. These data

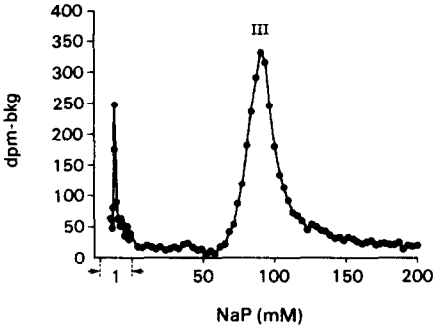

Fig. 3. IEC profile of corticoid receptor I sites. Steroid–receptor complexes were prepared and IEC conducted in the same manner as that described in Fig. 1A, except cells were incubated with 2 nM [^{3}H]Aldo in the presence of 40 nM nonradioactive dexamethasone to correspond with previous binding experiments [6]. Only peaks I and II were displaced by DM.

together with the specificity data of Table 1 and Fig. 2 indicate that peak III corresponds to corticoid receptor I sites, which can be physically separated from corticoid receptor II sites by IEC.

Stability of corticoid receptor I and corticoid receptor II sites during IEC

If K_d and B_{max} are known, then at any concentration of ligand, one can solve for the steroid–receptor complex concentration. For example, at 5 nM [^{3}H]Aldo, 90% of type I sites and 33% of type II sites are occupied; since the ratio of type I to type II sites is 1:5, then 35% of the total bound sites are type I and 65% are type II. If these data are then compared with the percentage of radioactivity in peaks I and II for corticoid receptor II sites and peak III for corticoid receptor I sites, the relative stability of the two receptor classes during IEC can be determined. These results are summarized in Table 2 and indicate that when [^{3}H]Aldo was the ligand, both sites are present in the same proportion following IEC as that determined by previous gel filtration binding assays. Therefore, neither receptor site was lost or recovered disproportionately during these experiments.

DISCUSSION

IEC has been used to separate corticoid I and corticoid II receptors in cultured aortic smooth muscle cells. Three peaks of radioactivity are eluted with

Table 2. Proportion of type I and type II steroid–receptor complexes: comparison between binding assay and IEC data

Experiments	Incubation conditions	Predicted by binding statistics % Type I/% Type II[a]	Eluted from IEC column % Type I/% Type II[b]
1	5 nM [^{3}H]Aldo	35/65	41/59
2	5 nM [^{3}H]Aldo	35/65	44/56
3	10 nM [^{3}H]Aldo	28/72	38/62

[a]Binding statistics used were: K_dI = 0.5 nM, B_{max}I = 100 mol × 10^{-18}/μg DNA: K_dII = 10 nM; B_{max}II = 500 mol × 10^{-18}/μg DNA [5].
[b]Estimated geometrically as area under curve from IEC profile.

increasing phosphate concentration when [^{3}H]Aldo or [^{3}H]B are used to label steroid–receptor complexes. The steroid-specificities of peaks I and II are identical to that reported for corticoid receptor II sites and the steroid-specificity of peak III is identical to that reported for corticoid receptor I sites [5]. In addition, the proportion of both sites eluted from IEC columns was similar to that applied as determined from the binding statistics. No sites with the same hierarchy of affinity as corticosteroid binding globulin have been identified in cultured aortic cells by a gel filtration steroid binding assay or by IEC.

Aortic corticoid receptor I sites have the same intrinsic specificity as renal mineralocorticoid type I receptors and aortic corticoid receptor II sites have the same specificity as classical glucocorticoid type II receptors [3]. The anion-exchange data presented here are in agreement with that reported for type I and type II sites in other tissues and cells. In previous studies, using a different starting and eluting buffer composition, renal type I mineralocorticoid receptors also elute as a single peak on IEC and are retained more than renal type II receptors [16]. Type II glucocorticoid receptors typically elute as two peaks on IEC in cells [17] and tissues [18, 19]. It is not known if the two forms represent different subunits or activated and unactivated forms of the same receptor or even the native structure and a proteolytic product [12, 20].

On the basis of protein concentration, vascular type I receptors are purified 15-fold by IEC. If the type I sites can be stabilized for further manipulation, then IEC will be useful in purification of type I receptors from a vascular source just as it has been useful in the purification of molybdate-stabilized renal mineralocorticoid receptors [21].

Sherman *et al.*[20] have shown differences in ion exchange properties of molybdate-stabilized glucocorticoid receptors in fresh cytosols from rat kidney and liver that may result from differences in endogenous proteolytic enzymes. Protease-rich cytosols may account for the multiple mineralocorticoid- and glucocorticoid-specific peaks described by Agarwal[11] using a similar IEC method to the one described here. The fewer peaks obtained in these studies may reflect less protease activity in vascular smooth muscle cells or an increased stability of sites due to the inclusion of 20% glycerol in IEC buffers.

In conclusion, the results of IEC of corticoid receptors in cultured aortic cells have verified previous binding assays and competition studies. Vascular smooth muscle cells contain both type I and type II corticoid receptor sites. Separation of these sites, which has been accomplished by IEC was necessary for further studies on the role of adrenal steroids and type I and II sites in vascular smooth muscle cell physiology.

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