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Solubilization and partial purification of the thiazide diuretic receptor from rabbit renal cortex

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This study was designed to solubilize, characterize and begin to purify the thiazide-sensitive Na/Cl transporter from mammalian kidney. Metolazone, a thiazide-like diuretic drug, binds to receptors in rat renal cortex closely related to the thiazide-sensitive Na/Cl transport pathway of the renal distal tubule. In the current study, [^{3}H]metolazone bound to receptors in rabbit renal cortical microsomes. The portion of [^{3}H]metolazone binding that was inhibited by hydrochlorothiazide reflected binding to a high-affinity class of receptor. The affinity (K_d 2.0 ± 0.1 nM) and number (B_{max} = 0.9 ± 0.4 pmol/mg protein) of high-affinity receptors in rabbit renal cortical membranes were similar to values reported previously for rat. When proximal and distal tubule fragments were separated by Percoll gradient centrifugation, receptors were restricted to the fraction that contained distal tubules. When compared with cortical homogenates, receptor density was enriched 12-fold by magnesium precipitation and differential centrifugation. The zwitterionic detergent CHAPS solubilized 25–35% of the receptors (at 6 mM). Chloride inhibited and Na stimulated binding of [^{3}H]metolazone to solubilized high-affinity receptors. The receptors could be purified significantly by hydroxyapatite chromatography and size exclusion high performance liquid chromatography (HPLC). The combination of magnesium precipitation and differential centrifugation, hydroxyapatite chromatography, and size exclusion HPLC resulted in a 213-fold enrichment of receptors, compared to renal cortical homogenate. The current results indicate that thiazide receptors from rabbit kidney share characteristics with receptors from rat, and that rabbit receptors can be solubilized in CHAPS and purified significantly by hydroxyapatite chromatography and size exclusion HPLC.

Introduction

Thiazide diuretics inhibit the movement of Na and Cl across the apical membrane of mammalian distal convoluted tubule (DCT) cells [1]. These drugs appear to interact with a protein that couples the movement of Na and Cl across cell membranes, but which is distinguishable pharmacologically from the bumetanide-sensitive Na/K/2Cl cotransport pathway [2]. Beaumont and Fanestil [3] reported that a tritiated derivative of the thiazide-like diuretic metolazone binds to receptors in rat kidney. There is convincing evidence that the high-affinity [^{3}H]metolazone receptor is, or is a part of, the Na/Cl cotransport pathway itself. First, several thiazide and thiazide-like drugs inhibit binding of [^{3}H]metolazone to this receptor with a rank order potency that correlates with their clinical doses [3]; second, chloride inhibits and sodium stimulates binding to the receptor, suggesting that the drug competes with chloride at the anion site on the transporter [4]; third, receptors are located exclusively along short segments of the cortical nephron that may correspond to distal tubules [5]; finally, Fanestil and co-workers [6] and ourselves [7,8] have shown that the density of high-affinity [^{3}H]metolazone binding sites is altered by factors that also affect the NaCl transport capacity of distal tubules. Thus, high-affinity receptors for [^{3}H]metolazone appear to participate directly in the movement of Na and Cl across the apical membrane of distal tubule

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cells. The present experiments were designed to solubilize these receptors, to characterize the solubilized receptors, and to begin to purify them.

Methods

Preparation of membranes

Kidneys were removed rapidly from anesthetized rabbits and chilled immediately in iced homogenization buffer (Buffer I: 50 mM Tris phosphate (pH 7.4), (reagents were obtained from Sigma, unless otherwise noted)). The renal capsule and all medullary tissue were removed and the cortex was minced. Cortical tissue was homogenized in a tissue grinder (Potter Elvehjem type, Wheaton Instruments, Millvile, NJ) with ice-cold Buffer II (1 part tissue, 4 parts buffer, Buffer II = Buffer I + 0.5 mg/l leupeptin, 40 mg/l phenylmethylsulfonyl fluoride, 0.7 mg/l pepstatin, and 1 mM EDTA) at 2100 rpm using 20–25 passes. The cortical homogenate was used either to prepare cortical microsomes or for magnesium precipitation.

Cortical microsomes. The cortical homogenate was centrifuged for 10 min at $1000 \times g$ to remove large debris. The supernatant was collected and centrifuged at $48000 \times g$ for 20 min. The resulting pellet was resuspended in Buffer III (Buffer III = Buffer II + 50 mM sodium gluconate) and frozen at −70 °C. Each membrane preparation was derived from 4–8 rabbits.

Magnesium precipitation. Membranes were prepared using a technique modified from Booth and Kenney [9] and Aronson [10]. Renal cortical tissue was homogenized as above and precipitated for 1 h with 5 mM Mg (as the hemimagnesium salt of gluconic acid) in Buffer I. The homogenate was centrifuged for 10 min at $2300 \times g$ and the resulting supernatant was retained and centrifuged at $30000 \times g$ for 20 min. The pellet was resuspended in Buffer II with 5 mM Mg. The suspension was centrifuged at $3000 \times g$ for 10 min; the resulting supernatant was centrifuged for 20 min at $30000 \times g$. The pellet was again resuspended in Buffer II with magnesium and centrifuged for 10 min at $3600 \times g$. The supernatant was then centrifuged for 20 min at $30000 \times g$. The pellet was resuspended in Buffer II without Mg and centrifuged again at $30000 \times g$ for 20 min. The final pellet was resuspended in Buffer III to a final protein concentration of approximately 20 mg/ml and either stored at −70 °C or solubilized.

Percoll gradient centrifugation. The technique for separating proximal and distal tubules was modified from Brunette and co-workers [11,12]. Kidneys were removed and decapsulated as above. Instead of mincing and homogenizing, however, slices of cortex (1–3 mm thick) were stirred constantly at 37 °C in 200 ml Krebs-Henseleit buffer (Sigma) with 2 mM $CaCl_2$, 1 mg/ml collagenase (Type V, Sigma), and 2 mg/ml bovine serum albumin. The mixture was bubbled continuously with 95% O_2/5% CO_2. After 20 min, 200 ml of ice-cold Krebs-Henseleit (with $CaCl_2$, without collagenase or BSA) were added to stop digestion. The reaction mixture was then placed on ice for 5 min.

The rest of the preparation was carried out at 4 °C. The mixture was filtered through a tea strainer to remove large fragments. The suspension was washed three times by centrifugation for 2 min at $150 \times g$ in Krebs-Henseleit buffer. The final pellet containing tubule fragments was resuspend in 50% Percoll, 50% 2X Krebs-Henseleit (with 2 mM $CaCl_2$), previously bubbled with 95% O_2/5% CO_2. The mixture was centrifuged for 30 min at $28000 \times g$. Three major bands were visible following centrifugation; a fluffy white band in the most superficial layer, which contains distal tubules (confirmed by microscopy); a red tinged band just below this, which contains glomeruli and some tubules; and a thick slightly yellow band near the bottom of the gradient which contains proximal tubules (confirmed by microscopy). The locations of these tubule fragments following Percoll gradient centrifugation have been confirmed using enzyme markers [12].

Membranes from the top fluffy (distal) band and the bottom (proximal) band were then prepared using a magnesium precipitation technique. Each band was diluted 50:50 with Krebs-Henseleit buffer and washed three times by centrifugation, first at $1000 \times g$ 1 min, then at $150 \times g$ for 1 min to remove Percoll. The final pellets were resuspended in Buffer II and homogenized as above. The homogenate was precipitated in 12 mM $MgCl_2$ for 10 min and then centrifuged at $1000 \times g$ for 5 min. The resulting supernatant was centrifuged at $45000 \times g$ for 20 min. The pellet was washed twice in homogenizing buffer to remove any Cl and suspended in Buffer II.

Solubilization. Membranes were solubilized by stirring constantly at a final protein concentration of 3 mg/ml for 1 h in Buffer III with CHAPS ((3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) at 4 °C. Unsolubilized material was removed by centrifugation at $48000 \times g$ for 1 h. Solubilization was documented by showing that [^{3}H]metolazone binding sites remained in supernatant following $100000 \times g$ for 1 h and also passed through a 0.22 μm filter. The supernatant was frozen at −70°C. Because maximal solubilization was achieved using 6 mM CHAPS (see Results, below), solubilization was routinely performed at this concentration.

Radioligand binding

[^{3}H]Metolazone (specific activity, 16.6 Ci/mmol) was custom synthesized by Amersham (Arlington Heights, IL) according to a protocol described by Beaumont and Fanestil [3], from a precursor, 7-chloro-3,4-dihydro-2-methyl-4-oxo-3-*o*-tolyl-6-quinazolinesulfonanamide, generously supplied by Fisons Pharmaceuticals

(Rochester, NY). Unless otherwise noted, binding studies were performed in Buffer III which contained 50 mM sodium Gluconate, 0 Cl and 0 K. 50 μM acetazolamide was present in all reaction mixtures to inhibit binding to carbonic anhydrase as described [3]. As discussed below, parallel incubations with and without $2 \cdot 10^{-5}$ M hydrochlorothiazide were used to determine high-affinity [^{3}H]metolazone binding. Binding to intact membranes was performed by incubating [^{3}H]metolazone with membranes at a final reaction volume of 1 ml (final protein concentration 0.5 mg/ml) at 4°C for 4 h. Bound metolazone was separated from free by vacuum filtration (Millipore DAWP 293 25, pore size 0.65 μm) using filters presoaked in 0.3% polyethylenimine to reduce non specific binding [3]. The filters were then washed with 2.0 ml ice-cold buffer four times, placed in scintillation vials, and counted in a liquid scintillation counter using Optifluor (New England Nuclear) as the scintillation medium. The amount of [^{3}H]metolazone that adhered nonspecifically to filters was determined by passing appropriate concentrations of [^{3}H]metolazone over filters in the absence of protein. These values were subtracted. All determinations were done in duplicate.

Binding to solubilized proteins was determined in a similar manner except that the final protein concentration was approximately 1 mg/ml and the final reaction volume was 130 μl. Separation of bound from free [^{3}H]metolazone was determined using the procedure of Penefsky [13]. Sephadex G-50 was swelled overnight in Buffer III with 6 mM CHAPS. Plastic syringes (3 ml) were fitted with polyester fiber and filled to 2.5 ml with Sephadex. Columns were dried prior to use by spinning at 2000 rpm for 2 min. 130 μl of reaction mixture were applied and the columns were centrifuged for 2 min at 2000 rpm. Radioactivity in the eluate was measured by liquid scintillation counting using Optifluor as the scintillant. Pilot experiments indicated that free metolazone was adequately separated from bound using this procedure. Determinations were done in duplicate or triplicate, depending on the quantity of protein available.

The time course of [^{3}H]metolazone binding was studied at 4°C. High-affinity binding as a function of time was determined at a [^{3}H]metolazone concentration of 12 nM. The time at which steady state was reached was determined by showing that the amount of bound [^{3}H]metolazone varied by $< 2\%$ during three consecutive measurements. After steady state binding was achieved, $2 \cdot 10^{-5}$ M HCTZ or buffer was added to the incubation mixture and dissociation was determined as a function of time. Data analysis calculations (taken from Ref. 14) are shown in the figure legend.

The effects of Na, K and Cl on binding of [^{3}H]metolazone were compared by incubating solubilized proteins with 10 nM [^{3}H]metolazone in Buffer III with 6 mM CHAPS. This buffer contains no Na, Cl or K. Stock solutions (400 mM of sodium gluconate, potassium gluconate, or NaCl) were added to give final ion concentrations of 0, 25, 50, 75, and 100 mM. The final reaction volume was adjusted to 160 μl. The proteins were incubated as above and the amount of HCTZ inhibitable binding was assessed after 8 h of incubation at 4°C.

Protein purification

Hydroxyapatite chromatography. Hydroxyapatite (Bio-Gel HT, Bio-Rad, bed volume 5 ml) was loaded onto columns (Econo columns, Bio-Rad) and washed with 100 bed volumes of 1 mM sodium phosphate (pH 7.4) containing 6 mM CHAPS and proteinase inhibitors. Three ml of solubilized protein (1–1.5 mg protein/ml) were applied to columns and eluted with 10–100 mM isohydric gradient of sodium phosphate with 6 mM CHAPS (total volume 25 ml). Fractions were incubated with [^{3}H]metolazone as described above, free [^{3}H]metolazone was removed by Sephadex filtration, and samples were counted in a liquid scintillation counter using Optifluor. Because initial experiments indicated that high-affinity [^{3}H]metolazone receptors appeared in the early flow through, in some subsequent experiments, the receptors were eluted using only 10 mM sodium phosphate with CHAPS.

For some experiments, [^{3}H]metolazone was bound to solubilized proteins prior to hydroxyapatite chromatography. For this technique, proteins were incubated with [^{3}H]metolazone as described above and free [^{3}H]metolazone was removed by rapid filtration over Sephadex. The [^{3}H]metolazone-protein complexes were then applied to hydroxyapatite columns and eluted as above. Prebinding was possible because [^{3}H]metolazone dissociates from high-affinity receptors very slowly at 4°C ($t_{1/2}$ = 13.1 h, see Results) and hydroxyapatite chromatography was complete within 2 h. The validity of this approach was confirmed by showing that results were similar whether samples were bound prior to or after hydroxyapatite chromatography. The entire procedure was done at 4°C.

Size exclusion chromatography. Solubilized proteins were applied to a Zorbax GF-450 size exclusion column connected to a Waters HPLC system prequilibrated with Buffer III with 6 mM CHAPS. The running buffer was Buffer III with 6 mM CHAPS. The flow rate was 0.5 ml/min. Fractions were collected each minute at 25°C. Specific binding activity was determined by incubating collected fractions with 12 nM [^{3}H]metolazone, removing unbound metolazone as described above and counting in a liquid scintillation counter. The protein concentration in each sample was determined. Molecular weight markers are described in the figure legends.

CHAPS-polyacrylamide gel electrophoresis. A 5 to

10% polyacrylamide gradient gel was prepared. The gradient was formed by combining two solutions using a gradient maker. The first solution contained 2.2 ml of 30% acrylamide/0.8% Bis, 7.7 ml H_2O, 3.3 ml of 1.5 M Tris base (pH 8.8) with 24 mM CHAPS. The second solution contained 4.4 ml of 30% acrylamide/0.8% Bis, 5.5 ml H_2O, and 3.3 ml of 1.5 M Tris base with 24 mM CHAPS. Polymerization was initiated by adding 100 μl of 10% ammonium persulfate and 5 μl TEMED to each. The stacking gel contained 3% acrylamide, and a final CHAPS concentration of 6 mM. The pH was 6.8.

2 ml of solubilized protein was incubated with [^{3}H]metolazone for 6–12 h, as described above. Free [^{3}H]metolazone was removed by filtration over Sephadex and the proteins were diluted 1:3 in treatment buffer which contained 2.5 ml of 0.5 M Tris phosphate (pH 6.8), 2.5 ml of 60 mM CHAPS, 2.5 ml glycerol, 2.5 ml H_2O, and Bromphenol blue and loaded at 500 μg of protein per lane. The gel was run for 12 h at constant current (200 mA) continuously cooled by water at 4°C. For determination of radioactivity, the gel was sliced into 5-mm fractions. Each slice was digested overnight in 30% H_2O_2 at 78°C and the radioactivity was determined by liquid scintillation counting using Optifluor as the scintillation medium. Samples were counted only after the initial artifactual counts resulting from digestion had disappeared. Parallel lanes, loaded in an identical fashion were stained with silver.

Protein determination

Protein was determined by the method of Lowry et al. [15] or, for solubilized proteins, by a modified method of Bradford (Bio-Rad Protein Assay). CHAPS interferes with protein determination using this method. Thus, 6 mM CHAPS was included in all blank solutions, a procedure which was shown to correct adequately for interference. Albumin was used as the standard for the Lowry measurements. Use of albumin as the standard for the Bradford method overestimates protein concentration compared with a battery of protein standards (see Bio-Rad Protein Assay Package Insert). Thus, ovalbumin was used as a reference standard for Bradford assays.

Results

Fig. 1 shows results of equilibrium binding studies using cortical microsomes from rabbits. The portion of [^{3}H]metolazone binding that is sensitive to HCTZ is a saturable function of [^{3}H]metolazone concentration (Fig. 1A), and is linear by Scatchard analysis (Fig. 1B). The equilibrium dissociation constant of the HCTZ sensitive component of [^{3}H]metolazone receptors ('high-affinity receptors') is 2.0 ± 0.1 nM and the maximum number of binding sites is 0.9 ± 0.4 pmol/mg protein (n = 3).

Fig. 2A shows that magnesium precipitation and differential centrifugation increased the number of high-affinity binding sites by 3-fold (B_{max} = 2.5 pmol/mg protein) compared with cortical microsomes (0.9 pmol/mg protein), and 10-fold compared with crude cortical homogenate (B_{max} = 0.3 pmol/mg protein). Because the density of binding sites was highest in membranes prepared by magnesium precipitation, this preparation was used as starting material for all solubilization and purification experiments. To determine whether high-affinity binding sites were located predominantly in proximal or distal tubules, Percoll gradient centrifugation was used to prepare tubule populations enriched in proximal and distal fragments.

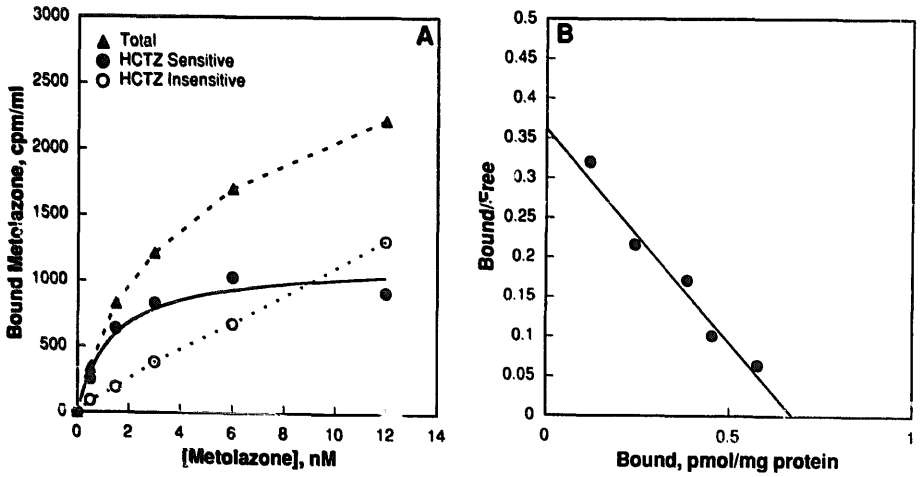

Fig. 1. [^{3}H]Metolazone binding to rabbit cortical microsomes. (Panel A) Specific binding as a function of [^{3}H]metolazone concentration (from 0.6 to 12 nM). ▲, Total binding; ○, residual binding in the *presence* of $2 \cdot 10^{-5}$ M cold hydrochlorothiazide; ● the portion of binding that is inhibited by $2 \cdot 10^{-5}$ M hydrochlorothiazide (the difference between total and residual binding). (Panel B) Scatchard plot of HCTZ-sensitive portion of [^{3}H]metolazone binding. Bound/Free is plotted in units of (pmol/mg protein)/(pmol/10^3 l).

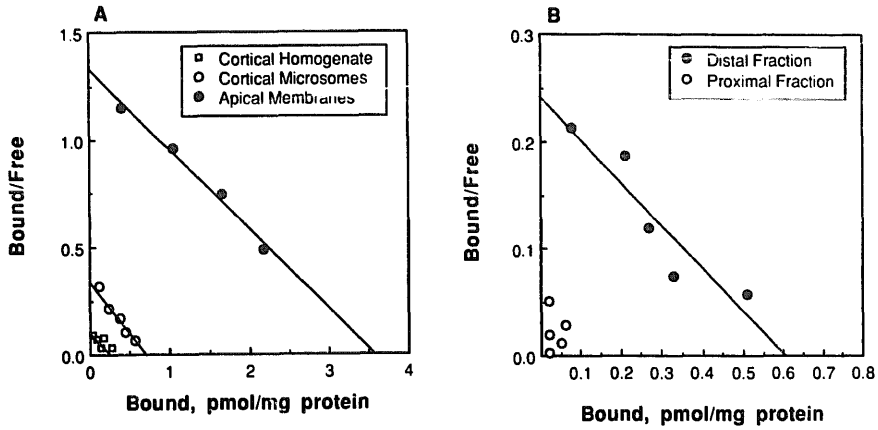

Fig. 2. Scatchard analysis of HCTZ-inhibitable [^{3}H]metolazone binding. (Panel A) ■, Crude cortical homogenate; ○, cortical microsomes; ●, apical membranes, prepared by magnesium precipitation and differential centrifugation. B_{max} values were greatest in apical membrane preparations and lowest in crude cortical homogenates. K_d values were not significantly different. Bound/Free is plotted in units of (pmol/mg protein)/(pmol/10^3 l). (Panel B) HCTZ-inhibitable [^{3}H]metolazone binding to membranes derived from proximal and distal fractions (representative of two separate preparations). Binding to the proximal fraction was minimal and was not fit significantly by linear regression analysis.

Fig. 2B shows that binding sites were present in distal tubules, but were absent from the proximal fraction.

The high-affinity [^{3}H]metolazone receptors were solubilized using the zwitterionic detergent CHAPS. Fig. 3 shows that binding sites were solubilized most efficiently at 6 mM CHAPS, at which concentration 25%–35% of high-affinity binding sites and 30% of the protein were present in the soluble fraction. CHAPS concentrations greater than 8 mM or less than 4 mM were associated with lower solubilization efficiencies. For all further experiments, membranes were solubilized at 6 mM CHAPS.

To quantitate the rate of binding of [^{3}H]metolazone to its receptor, high-affinity binding was determined as a function of time at 4°C (Fig. 4). Binding was saturable and half maximal at 52 ± 6 min ($n = 3$). The half-time for dissociation, determined by adding excess cold HCTZ ($5 \cdot 10^{-5}$ M) to a reaction mixture that had reached steady state (at 6 h), was 13.1 ± 1.9 h ($n = 3$). The kinetic data were used to calculate an association rate constant ($k_a = 9.0 \pm 1.9$ min^{-1}) and a dissociation rate constant ($k_d = 9.9 \pm 1.2$ min^{-1} M^{-1}). The equilibrium dissociation constant derived from these data ($K_d = k_d/k_a$) is 1 nM. The solubilized receptors were stable for several months when stored at −70°C; they retained >90% of binding activity after 24 h at 4°C.

Chloride inhibits and Na stimulates [^{3}H]metolazone binding to intact and solubilized receptors from rat [4,16]. To determine whether these ions alter [^{3}H]metolazone binding to solubilized receptors from rabbit, the effects of Na, K, and Cl on binding were compared. Fig. 5A shows that Cl reduces binding whereas Na stimulates it. Chloride appears to compete for binding with thiazides at the anion site on the Na/Cl cotransporter of the rat [4]. To determine whether Cl and [^{3}H]metolazone compete for the same binding site on the solubilized rabbit receptors, Scatchard analysis was performed in the presence and absence of 50 mM chloride (Fig. 5B). Chloride increased the K_d significantly from 7 ± 1 nM to 48 ± 10 nM ($n = 3$, $P < 0.05$) without affecting the B_{max} (Cl: 3.1 ± 1.4 vs. 0 Cl: 2.5 ± 0.7 pmol/mg protein).

Solubilized [^{3}H]metolazone binding sites were purified using hydroxyapatite chromatography and size exclusion HPLC. When solubilized membranes were applied to hydroxyapatite columns, the high-affinity [^{3}H]metolazone receptors eluted in the early flow through, together with a small peak of protein, as shown in Fig. 6. 25–35% of the applied binding sites were recovered in fractions 2–4, whereas only 8–12% of the applied protein was recovered in these same fractions. The specific binding activity was enriched by

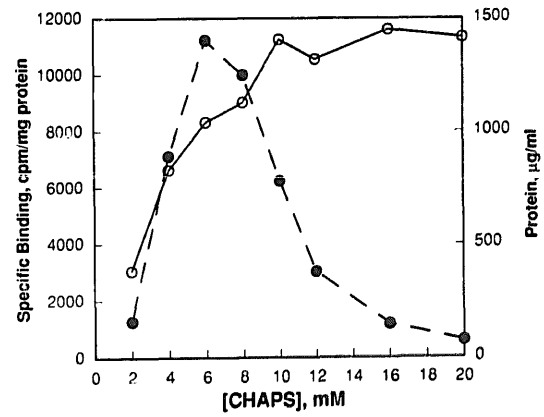

Fig. 3. Effects of CHAPS concentration on solubilization of protein and high-affinity binding sites. Proteins were incubated at a final concentration of 3 mg/ml at 12 nM [^{3}H]metolazone. ●, Specific binding in cpm/mg protein; ○, protein concentration in µg/ml. Data are representative of two experiments.

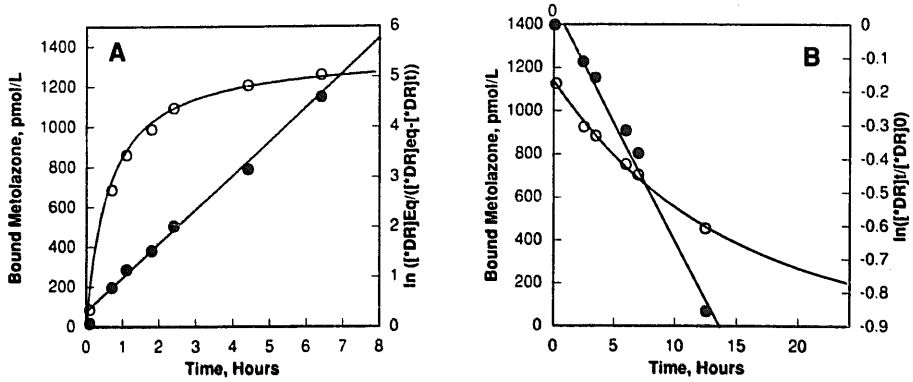

Fig. 4. (Panel A) High-affinity [^{3}H]metolazone binding to solubilized proteins versus time (representative of three experiments). ○, The concentration of [^{3}H]metolazone bound to high-affinity receptors in pmol/l as a function of time. To obtain k_a, the association rate constant, the rate of binding was plotted (●) as $\ln([*DR]_{eq}/([*DR]_{eq}-[*DR]_t))$, where $[*DR]_{eq}$ is [^{3}H]metolazone bound to high-affinity receptors at steady state (in this case 12 h) and $[*DR]_t$ is the concentration of [^{3}H]metolazone bound to high-affinity receptors at time = t. The slope of this line is $k_{obs} = k_a + k_d = 0.0012$/min (determined by linear regression analysis). (Panel B) Time course of displacement of [^{3}H]metolazone by $2 \cdot 10^{-5}$ M HCTZ. Solubilized receptors were incubated with [^{3}H]metolazone until steady state was reached after which $2 \cdot 10^{-5}$ M HCTZ was added to the reaction mixture. ○, The concentration of [^{3}H]metolazone bound to high-affinity sites. To obtain k_d, the dissociation rate constant, the $\ln([*DR]_t/[*DR]_0)$ was plotted versus time (●), where $[DR*]_0$ is the concentration bound at time = 0. k_d is the (negative) slope of the line determined by linear regression analysis [14]. k_a can then be determined from k_{obs} and the value for k_d [14].

3–7-fold in the peak fraction (see Table I). Fraction 3 eluted from the hydroxyapatite columns was applied to a Zorbax GF-450 column. Fig. 7 shows that [^{3}H] metolazone binding sites eluted in a broad peak of approximately 346 000 (apparent molecular weight).

SDS-polyacrylamide gel electrophoresis revealed several visible bands that were enriched by the purification procedure. Because radioligand binding was labile when receptors were incubated in SDS under either reducing or non reducing conditions, we attempted to separate proteins in a non denaturing gel using CHAPS as the detergent. Proteins eluted from hydroxyapatite columns were prelabelled with [^{3}H] metolazone and separated by CHAPS-PAGE. Although this method does not separate proteins strictly according to molecular weight, significant protein separation was achieved using this technique (Fig. 8). The [^{3}H]metolazone binding sites ran as a relatively narrow band in the first 2 cm of the gel.

Table I compares the the specific binding activity of the membrane preparations and partially purified proteins. Compared with cortical homogenate, binding sites were enriched 14-fold in solubilized apical membranes. The combination of hydroxyapatite chromatog-

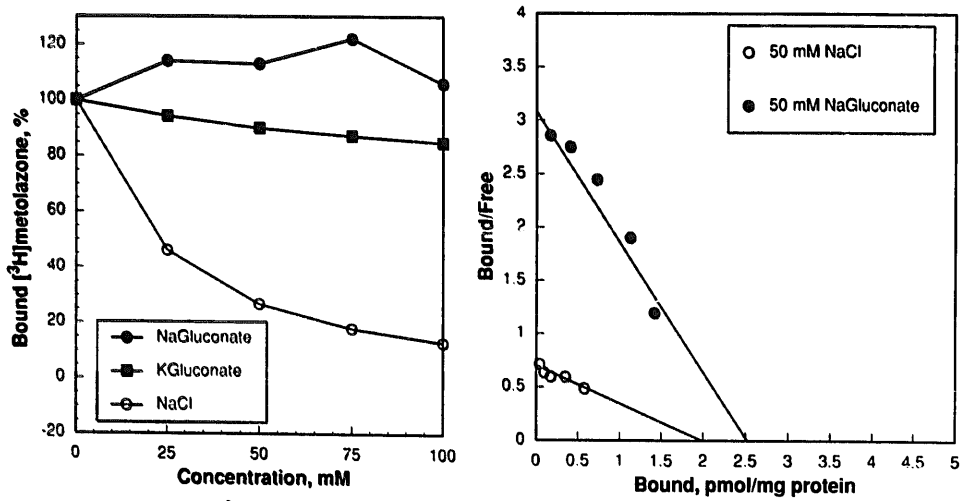

Fig. 5. Effects of ions on HCTZ-inhibitable [^{3}H]metolazone binding. (Panel A) Effects of sodium gluconate, potassium gluconate, and NaCl on high-affinity binding, expressed as percent compared with binding in the absence of Na, K, and Cl ($n = 2$). Reactions were carried out at 10 nM [^{3}H]metolazone. (Panel B) Effects of 50 mM chloride on HCTZ-inhibitable [^{3}H]metolazone binding. Scatchard plot shows binding in the presence of 50 mM sodium gluconate (0 Cl, ●) and 50 mM sodium Cl (○). Ionic strength and pH were the same under both conditions. Bound/Free is given in units of (pmol/mg protein)/(pmol/10^{-4} l). Data are representative of three independent experiments.

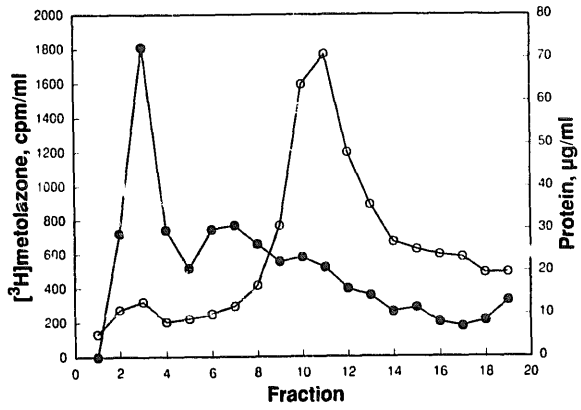

Fig. 6. Hydroxyapatite chromatography of solubilized [^{3}H]metolazone receptors. Solubilized proteins were applied to columns and eluted with a 10–100 mM isohydric gradient of sodium phosphate. 1 ml fractions were collected. ○, Protein concentration, in µg/ml; ●, bound [^{3}H]metolazone in cpm/ml.

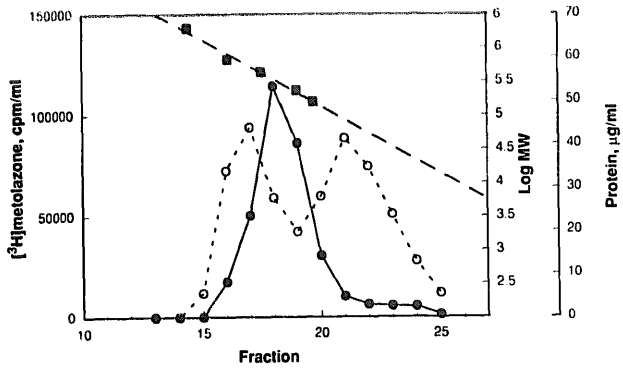

Fig. 7. Results of size exclusion HPLC. ●, The concentration of bound [^{3}H]metolazone in cpm/ml; ○, protein concentration, in µg/ml. Molecular weight markers, indicated by the filled squares are, blue dextran (2000000), thyroglobulin (669000), ferritin (440000), catalase (232000), and aldolase (158000). The dashed line is the least-squares fit for the molecular weight standards ($y = 8.6–0.17x$).

raphy and size exclusion HPLC resulted in a further purification of 16-fold for a final increase in specific binding activity of greater than 200-fold, compared with crude membranes.

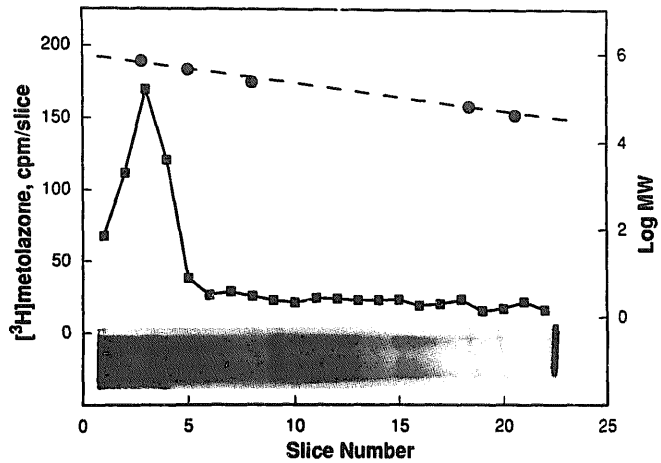

Fig. 8. CHAPS-PAGE. ■, Radioactivity in each 5 mm slice of polyacrylamide gel. ●, The positions of molecular weight standards plotted versus the log of the molecular weight. The standards are thyroglobulin (669000), ferritin (440000), catalase (232000), bovine serum albumin (67000), ovalbumin (43000). A parallel lane (shown at the bottom) was loaded with bound protein and silver-stained. The data in this figure are representative of four similar experiments. Free metolazone (run in a parallel lane) ran near the dye front.

Discussion

The majority of NaCl transport across distal tubules of the mammalian kidney traverses a pathway in the luminal membrane that is sensitive to thiazide diuretics [17,18]. Functional studies suggest that the movements of Na and Cl are directly coupled by this transport pathway and occur in an electroneutral fashion [2,17,19]. Recent radioligand studies are consistent with the hypothesis that both Na and Cl bind to sites on the same transport protein and that thiazide and thiazide-like diuretics bind at the anion site of the transporter [4]. [^{3}H]Metolazone binds to receptors in rat kidney cortex, one class of which probably represents the thiazide-sensitive transport pathway, or a portion thereof [3]. The present experiments were designed to solubilize, characterize and begin to purify the high-af-

TABLE I

Purification table

Data are given per kidney. Enrichment = specific activity/specific activity of cortical homogenate. Percentage yield compares the total number of binding sites with the number of binding sites in cortical homogenate.

Material	Total protein (mg)	Specific activity (pmol/mg protein)	Enrichment (x)	Total binding sites (pmol)	Yield (%)
Cortical homogenate	1000	0.3	–	300	100
Cortical microsomes	204	0.9	3	184	41
Apical membranes	16	2.5	8	40	18
Solubilized protein	6	4.2	14	25	8
Hydroxylapatite	0.3	15.2	50	5	2
Size exclusion HPLC	0.03	64	213	2	0.6

finity class of [^{3}H]metolazone receptors from rabbit kidneys. The results indicate that [^{3}H]metolazone binds to receptors from rabbit kidney cortex that are similar to those described previously in rat. When solubilized using the detergent CHAPS, the receptors retain the binding properties of native receptors, and can be purified significantly by hydroxyapatite chromatography and by size exclusion HPLC.

Receptors for thiazide diuretics, which are present in rat renal cortex, have properties suggesting that they participate directly in NaCl transport [3,6–8]. In the rat, [^{3}H]metolazone binds to three classes of receptors; carbonic anhydrase, low-affinity receptors (which can be blocked by calcium channel antagonists), and high-affinity receptors (which can be blocked by thiazides) [3]. High-affinity receptors have binding properties that suggest they represent the physiologically relevant renal thiazide receptor [3]. In the present experiments, binding to carbonic anhydrase was blocked by including acetazolamide in the reaction mixture, even though this fraction comprises only 12% of total specific binding, at least in the rat renal cortex [3]. The portion of [^{3}H]metolazone binding that could be inhibited by excess cold HCTZ was linear by Scatchard analysis. This supports the hypothesis that HCTZ binds to a single class of receptors, as reported previously for the rat [3] and that the HCTZ-inhibitable portion of [^{3}H]metolazone binding can be used as an index of binding to high-affinity sites from rabbit kidney membranes. This technique has been used previously to measure binding to high-affinity receptors in rat [3].

Magnesium precipitation and differential centrifugation increased the density of receptors by approximately 10-fold, compared with crude cortical homogenate, and several fold over cortical microsomes, probably because this technique removes basolateral and intracellular membranes [20]. Similar brush border preparations have been shown to contain bumetanide-sensitive Na/K/2Cl pathways indicating that the preparation contains apical membranes from thick ascending limbs as well as proximal tubules [21]. Although the majority membrane fraction may be of proximal origin, magnesium precipitation and differential centrifugation probably enriches for apical membranes from distal tubules because DCT cells posses apical microvilli [22]. The absence of detectable NaCl cotransport in membranes from rabbits prepared by magnesium precipitation [23] may reflect inactivation of Na/Cl transporters by hypoxia [24]. To confirm that distal tubules are the sites in which high-affinity [^{3}H]metolazone receptors are located, Percoll gradient centrifugation was used to separate proximal and distal tubule fragments. This technique, frequently used for primary cell culture, generates relatively pure suspensions of proximal and distal cells. The results of bindings studies indicate that high-affinity [^{3}H]metolazone receptors are located almost exclusively in distal tubules.

Between 25% and 35% of receptors were solubilized using the zwitterionic detergent CHAPS, under the current conditions, together with approximately 30% of the protein. The percentage of solubilization in the current experiments is similar to that achieved by Luo et al. using Triton X-100 in experiments using rat thiazide receptors [16] In those experiments, however, CHAPS and other detergents did not solubilize rat receptors significantly. The different sensitivities of rat and rabbit thiazide receptors to solubilization conditions may reflect differences in structural properties of receptors from the two species, or differences in experimental techniques; Luo used glass fiber filters to separate bound from free [^{3}H]metolazone; the current experiments used Sephadex G-50. Further, Luo and coworkers used a dilution technique to reduce detergent concentrations prior to determination of binding [16].

The current results indicate that the binding properties of high-affinity [^{3}H]metolazone receptors remain essentially unaltered by solubilization. The equilibrium dissociation constant (K_d) of solubilized thiazide receptors is similar to values derived from unsolubilized membranes. Further, Cl inhibited and Na stimulated binding of [^{3}H]metolazone to solubilized receptors from rabbits, results qualitatively similar to those reported previously for receptors from rat kidney [4]. Based on interactions of Cl and Na with rat thiazide diuretic receptors, it was postulated that Cl and [^{3}H]metolazone compete for the same site on the NaCl transporter [4]. The current data suggest that [^{3}H]metolazone competes with chloride for binding in the rabbit as well.

Partial purification of the receptors was achieved using hydroxyapatite chromatography and size exclusion HPLC. Hydroxyapatite has been used to purify a variety of proteins [25], including several membrane associated transport proteins [26,27] and does not separate proteins primarily on the basis of molecular size. For this reason, size exclusion HPLC was chosen as a complementary technique. The combination of hydroxyapatite chromatography and size exclusion HPLC increased the specific binding activity by 16-fold and provided an estimate of molecular weight (near 350000). It must be emphasized that this estimate reflects the behavior of receptors in detergent micelles and does not necessarily correlate with the molecular mass of individual proteins or protein subunits. Nevertheless, together with the results of non denaturing CHAPS-PAGE, the results of these experiments suggest that the native thiazide receptors are large [28].

In summary, thiazide diuretic receptors were shown to be present in cortical membranes from rabbit kidney. The receptors were located primarily in distal tubules and their density could be enriched by magne-

sium precipitation and differential centrifugation. The receptors were soluble in the zwitterionic detergent CHAPS and retained the radioligand binding properties of native receptors when solubilized. They could be purified significantly using hydroxyapatite chromatography and size exclusion HPLC. When compared with crude cortical homogenates, the combination of magnesium precipitation, solubilization, hydroxyapatite chromatography and size exclusion HPLC increased the specific binding activity of high-affinity thiazide receptors more than 200-fold.

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