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INHIBITION OF BINDING OF [^{3}H]METOLAZONE TO RAT KIDNEY MEMBRANE BY STILBENE DISULFONATES

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Abstract—Thiazide diuretics inhibit an electroneutral chloride and sodium reabsorptive transport pathway in the renal distal convoluted tubule. [^{3}H]Metolazone binds with high affinity to the drug recognition site on the thiazide-sensitive ion transporter. The molecular nature of this transporter is currently unknown. This report examines whether stilbene disulfonates [4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS)], agents that have been used to identify other anion-transporting proteins, could be useful in the identification of the thiazide receptor. We found that high concentrations of DIDS or SITS were required to inhibit binding of [^{3}H]metolazone to rat kidney membranes, with apparent IC_{50} values of 380 and 940 μM, respectively. The inhibition was due to a decrease in the number of binding sites without alteration in the affinity of the binding. The inhibition was not reversible, as judged by the inability of the inhibition to be reversed by removal of the DIDS or SITS from the membranes prior to equilibration with [^{3}H]metolazone. Addition of 100 mM NaCl to the incubation medium did not protect the [^{3}H]metolazone binding site from inhibition by DIDS. We infer that DIDS and SITS irreversibly inhibit binding of [^{3}H]metolazone by reaction with the thiazide receptor at a site other than the anion-transporting site.

Benzothiadiazine ("thiazide") diuretics are believed to act on an electroneutral sodium and chloride transport pathway in the renal distal tubule to inhibit the reabsorption of chloride and sodium from the tubular fluid [1–5]. We have used custom synthesized [^{3}H]metolazone, a quinazolinesulfonamide diuretic with a thiazide-like mechanism of action [6–8], to probe rat kidney for a binding site—or receptor—with the pharmacological characteristics [9] and renal tubular location [10] expected for the site at which thiazide-type diuretics exert their action. However, the specific membrane protein(s) and the functional group(s) on the protein(s) that participate in this chloride and sodium transport in the distal convoluted tubule have not been identified.

Disulfonic stilbene derivatives (e.g. DIDS† and SITS) have been used to identify specific protein and functional groups involved in anion transport in erythrocytes and other tissues [11]. In erythrocytes the function of the major anion transport protein, Band 3, can be modified with great selectivity by disulfonic stilbene derivatives [12–14]. More recently, Bastlein and Burckhardt [15] capitalized on the selectivity of DIDS for anion transport proteins to demonstrate that rat kidney membranes contain a sulfate transport activity that is irreversibly inhibited by DIDS. Since the inhibition by DIDS was prevented in the presence of 10 mM sulfate (i.e. it was "substrate protectable"), it was proposed that DIDS was suitable for affinity labeling studies on the sulfate transport system in renal basolateral membranes [15].

The experiments we report here were formulated to determine if stilbene disulfonates might prove similarly suitable for affinity labeling and identification of the chloride-transporting thiazide receptor. However, to be useful for this purpose the stilbenes must interact with the transporter and cause irreversible inhibition. Therefore, in this study we tested DIDS and SITS for actions as reversible and irreversible inhibitors of thiazide receptors in rat kidney. The results indicate that, although both DIDS and SITS produce inhibition of binding of [^{3}H]metolazone to the presumed Na–Cl co-transporter, relatively high concentrations are required. The inhibition produced by DIDS (IC_{50} = 380 μM) and by SITS (IC_{50} = 940 μM) met criteria for irreversibility. However, the inhibition was not decreased by the presence of NaCl, making it unlikely that the stilbenes react at the site on the protein involved in the recognition and transfer of chloride.

MATERIALS AND METHODS

Membrane preparation. Male Sprague–Dawley rats weighing 200–350 g were anesthetized with pentobarbital (50 mg/kg body wt). Kidneys were rapidly removed to ice-cooled 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] buffer (pH 7.4). (It should be noted that we primarily used a HEPES buffer in this report instead of the Tris buffer used in all prior reports from this laboratory [9, 10, 16–20] to avoid possible reaction between the amine on Tris and the stilbenes.) The kidneys were homogenized with a polytron (Brinkmann) and centrifuged at 1820 *g* for 5 min. The pellets were

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† Abbreviations: DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; and BSA, bovine serum albumin.

discarded and the supernatants were centrifuged at 45,650 *g* for 20 min. The resulting membrane pellets were resuspended in the HEPES buffer and centrifuged again at 45,650 *g* for another 20 min.

Binding assay. The final pellet containing the kidney membranes was resuspended with a polytron in 50 mM HEPES (pH 7.4) at a protein concentration of 0.7 to 1.0 mg/mL. As described previously [9], 10 μM acetazolamide and 10 μM nicardipine were added to the membrane suspension to block the binding of [^{3}H]metolazone to carbonic anhydrase and to occupy low-affinity binding sites, respectively. Membranes were usually incubated with 5 nM [^{3}H]metolazone in a volume of 1 mL in an ice bath for 4 hr. Where indicated, saturation analysis of the binding of [^{3}H]metolazone was conducted using six concentrations of [^{3}H]metolazone. Specific binding of [^{3}H]metolazone was defined as the binding displacable by addition of 100 μM hydroflumethiazide to a replicate set of assays [9]. The membrane receptors were collected by filtration through glass fiber filters (GF/B, Whatman), using a cell harvester (Brandel Instruments, Gaithersburg, MD) and washed with 12 mL of ice-cold 50 mM HEPES buffer. Filters were soaked for at least 1 hr before use with 0.3% polyethyleneimine. Radioactivity on the filters was extracted into scintillation fluid (Betaphase, Westchem, San Diego, CA) and counted at an efficiency of 52%. There was no specific binding of [^{3}H]metolazone to the filters. Protein was determined by the method of Bradford [21] with γ-globulin as the standard.

Inhibition by DIDS and SITS. Membranes were preincubated at 4° for 30 min in 50 mM HEPES (pH 7.4) with 0–2 mM DIDS or SITS prior to the 4 hr of equilibration with 5 nM [^{3}H]metolazone in the binding assay (see above). Where indicated, saturation analysis experiments were conducted to determine if DIDS altered the equilibrium dissociation constant (K_D) or the density of the binding sites (B_{max}) for [^{3}H]metolazone. In these cases, membranes were preincubated at 4° for 30 min with or without 1.0 mM DIDS (pH 7.4). Thereafter, the supernatants were diluted 30-fold and recollected by centrifugation. Membranes were then suspended in buffer for determination of the binding of [^{3}H]metolazone at six different concentrations of [^{3}H]metolazone. The data were analyzed according to the method of Scatchard as contained in the EBDA program of McPherson [22].

Irreversible inhibition by DIDS and SITS. Membranes were preincubated at 4° for 30 min in 50 mM Bicine [*N*,*N*'-bis(2-hydroxyethyl)glycine, pH 8.4], or 50 mM HEPES (pH 7.4), or 50 mM Bis-Tris [bis(2-hydroxyethyl) iminotris (hydroxymethyl) - methane, pH 6.4] with addition of 0 (control) or 1 mM DIDS or 1 mM SITS. Thereafter, the supernatants were diluted 30-fold into ice-cooled 50 mM HEPES (pH 7.4) without DIDS and SITS and centrifuged at 45,650 *g* for 20 min. The pellet was resuspended in HEPES buffer for measurement of [^{3}H]metolazone binding, as described above. In some experiments 1% bovine serum albumin (BSA) was included into the dilution buffer to trap tightly but not covalently bound DIDS [15], and centrifugation and resuspension steps were repeated once.

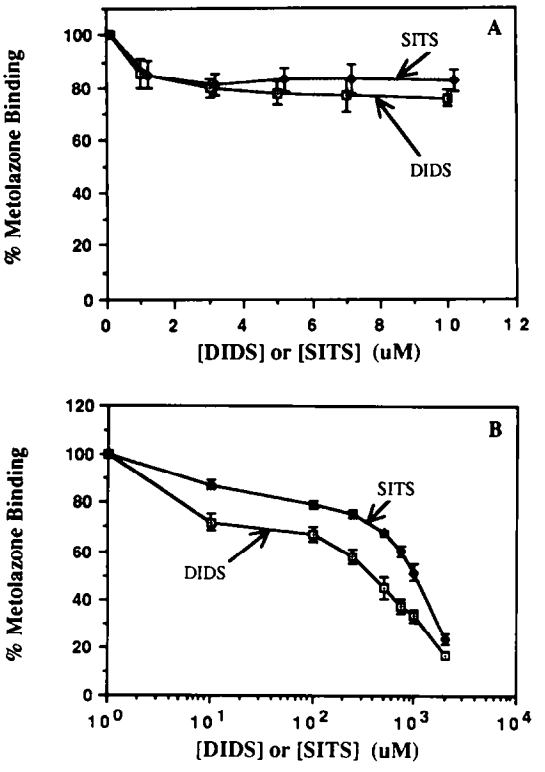

Fig. 1. Inhibition of binding of [^{3}H]metolazone to rat kidney membranes by DIDS and SITS. Membranes suspended in 50 mM HEPES (pH 7.4) were preincubated with different concentrations of DIDS and SITS at 4° for 30 min before addition of 5 nM [^{3}H]metolazone, as described in Materials and Methods. As shown, the symbols represent means ± SEM, N = 3. Panel A shows results at stilbene concentrations below 10 μM and panel B shows results at higher concentrations of stilbenes. The data in panels A and B were obtained concurrently on the same membrane preparations. Binding of [^{3}H]metolazone is expressed as the percent of the binding occurring in controls—that is, in the absence of DIDS and SITS—which was 0.33 ± 0.01 pmol/mg protein (mean ± SEM, N = 3).

Chemicals. DIDS and SITS were from Calbiochem (La Jolla, CA). [^{3}H]Metolazone (16 Ci/mmol) was custom synthesized by Amersham (Arlington Heights, IL) from a precursor supplied by Fisons (Rochester, NY) as described previously [9]. All other chemicals used in this study were of analytical grade.

RESULTS

Effects of DIDS and SITS on binding of [^{3}H]metolazone to rat kidney membranes. Initially, the effects of DIDS and SITS on binding of [^{3}H]metolazone to rat kidney membranes were assessed by incubating renal membranes with multiple concentrations of DIDS or SITS ranging from 1 μM to 2 mM at 4° for 30 min in 50 mM HEPES (pH 7.4). This was followed by a 4-hr equilibration with [^{3}H]metolazone. As shown in Fig.

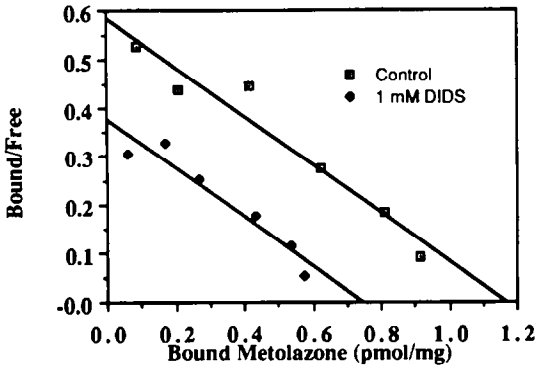

Fig. 2. Scatchard analysis of the effect of DIDS on the binding of [^{3}H]metolazone. Membranes were incubated without (symbols with dot) or with 1 mM DIDS (solid symbols) at pH 7.4 at 4° for 30 min, and then diluted 30-fold in ice-cold 50 mM HEPES buffer (pH 7.4). Membranes were collected by centrifugation, diluted in 50 mM HEPES buffer (pH 7.4), and equilibrated with six concentrations of [^{3}H]metolazone from 0.3 to 10 nM. The figure is one of three identical experiments. The means and standard error of the means for the three experiments are presented in Table 1.

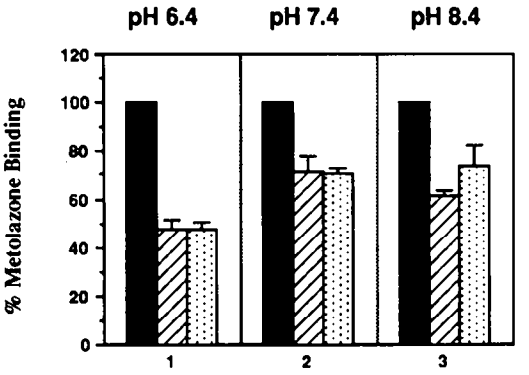

Fig. 3. Effects of pH and of BSA on inhibition of binding of [^{3}H]metolazone by DIDS. Membranes were incubated with DIDS (1 mM) at three different pHs at 4° for 30 min, and then diluted 30-fold in ice-cold 50 mM HEPES buffer (pH 7.4) with or without 1% bovine serum albumin. Results (mean ± SEM, N = 3) are expressed as percent of the control binding (solid columns) found in the absence of DIDS. Cross-hatched columns = DIDS treatment and dilution without BSA; stippled columns = DIDS + dilution with 1% BSA. The dilution was carried out once without BSA, and twice with BSA. The membrane pellets were resuspended in 50 mM HEPES (pH 7.4) for the binding assay (5 nM [^{3}H]metolazone). Control values for binding at pH 6.4, pH 7.4, and pH 8.4 were 0.35 ± 0.04, 0.34 ± 0.08 and 0.33 ± 0.07 pmol/mg protein, respectively (mean ± SEM, N = 3).

1A, at concentrations up to 10 μM, SITS inhibited binding of [^{3}H]metolazone by only 12.7 ± 1.99%. The inhibition produced by SITS increased to 32.2 ± 1.12%, 48.5 ± 3.35% and 76.0 ± 2.39% as the concentration of SITS was increased to 500 μM, 1 mM and 2 mM, respectively (Fig. 1B). DIDS was more effective than SITS as an inhibitor of the binding of [^{3}H]metolazone (Fig. 1). DIDS decreased binding by 27.9 ± 3.45%, 54.8 ± 4.49%, 66.6 ± 2.75% and 83.3 ± 1.69% at 10 μM, 500 μM, 1 mM and 2 mM, respectively. The IC_{50} values (i.e. concentrations of inhibitors that decreased binding of [^{3}H]metolazone by 50%) were 380 μM for DIDS, and 940 μM for SITS.

We next determined whether the decrease in binding of [^{3}H]metolazone was attributable to a change in the affinity of the receptor for metolazone or due to a loss or inactivation of binding sites for metolazone. An example of one of the three experiments in which saturation analysis of the binding of [^{3}H]metolazone was conducted is shown in Fig. 2. A summary of the results of the three experiments, presented in Table 1, indicates that

Table 1. Effect of DIDS on binding of [^{3}H]metolazone

Treatment	K_D (nM)	B_{max} (pmol/mg protein)
Control	2.08 ± 0.238	1.02 ± 0.103
DIDS (1 mM)	1.82 ± 0.193	0.655 ± 0.082*

Three membrane preparations were pretreated with or without 1 mM DIDS for 30 min prior to equilibration with six concentrations of [^{3}H]metolazone. Values are means ± SEM, N = 3.

* P = 0.007 control.

treatment with 1.0 mM DIDS for 30 min did not alter the equilibrium dissociation constant (K_D) of the binding but did decrease significantly the number of the binding sites (B_{max}) for [^{3}H]metolazone.

Irreversible inhibition by DIDS and SITS. Since both DIDS and SITS inhibited binding of [^{3}H]metolazone (Fig. 1), we tested experimental conditions used by prior workers [15] to determine if the inhibition produced by the treatment of membranes with 1 mM DIDS or SITS (at pH 6.4 for 30 min) was irreversible. In the first experiment for this purpose the membranes were reacted with DIDS or SITS at pH 6.4, 7.4 or 8.4 before unbound DIDS or SITS was removed by 30-fold dilution and subsequent recollection of the membranes in buffer at pH 7.4. Binding of [^{3}H]metolazone (conducted at pH 7.4) was still inhibited in all cases by the prior treatment with DIDS (Fig. 3) or SITS (Fig. 4). When the reaction of the membranes with DIDS or SITS had been conducted at pH 7.4 or 8.4, there was slightly less inhibition of binding of [^{3}H]metolazone than when the reaction had been conducted at pH 6.4. These results suggest that the inhibition may be irreversible, since the greater inhibition that was obtained at pH 6.4 could indicate that DIDS and SITS preferentially reacted with protonated amino groups on the thiazide receptor.

In the second experiment to test for the irreversibility of the inhibition, renal membranes were treated with DIDS or SITS for 30 min. The renal membranes were then diluted with 50 mM HEPES buffer containing 1% BSA to remove adherent but not covalently bound reagent. The

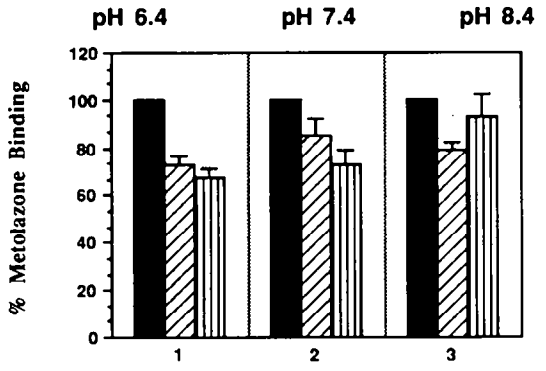

Fig. 4. Effects of pH and of BSA on inhibition of binding of [^{3}H]metolazone by SITS. Membranes were incubated with SITS (1 mM) at three different pHs at 4° for 30 min, and then diluted 30-fold in ice-cold 50 mM HEPES buffer (pH 7.4) with or without 1% bovine serum albumin. Results (means ± SEM, N = 3) are expressed as percent of the control binding (solid columns) found in the absence of SITS. Cross-hatched columns = SITS treatment and dilution without BSA; verticle striped columns = SITS + dilution with 1% BSA. The dilution was carried out once without BSA, and twice with 1% BSA. The membrane pellets were resuspended in 50 mM HEPES (pH 7.4) for the binding assay (5 nM [^{3}H]metolazone). Control values at the various pH levels are shown in the legend to Fig. 3.

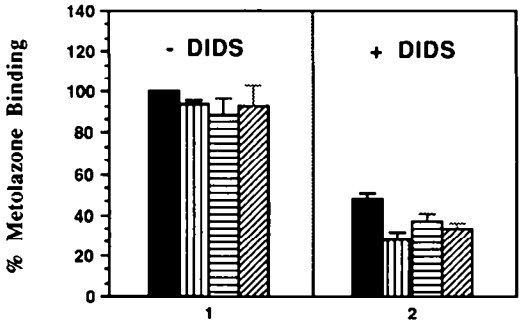

Fig. 5. Effects of NaCl and other salts on the irreversible inhibition of binding of [^{3}H]metolazone by DIDS. Membranes were incubated with DIDS (1 mM) at pH 6.4, 4°, in the absence or presence of 100 mM concentrations of various salts for 30 min. Solid columns = no added salt; verticle striped columns = NaCl; horizontal striped columns = Na_2SO_4; and cross-hatched columns = Cs_2SO_4. The membranes were then diluted in 30 vol. of pre-cooled 50 mM HEPES buffer (pH 7.4) and centrifuged. The washing step was repeated once. Binding of [^{3}H]metolazone by the membranes was examined at pH 7.4 (5 nM [^{3}H]metolazone). Control value in the absence of salt was 0.37 ± 0.3 pmol/mg protein. Values are means ± SEM, N = 3. Inhibition in the presence of NaCl + DIDS was significantly greater than in the absence of NaCl ($P < 0.001$).

results (Figs. 3 and 4) demonstrated that inclusion of BSA in the washing buffer did not reduce significantly the inhibition of binding of [^{3}H]metolazone produced by DIDS (DIDS still inhibited by 52.3%) or SITS (SITS still inhibited by 32.3%).

Effect of NaCl *and other salts on inhibition of binding of* [^{3}H]*metolazone to thiazide receptors by DIDS and SITS.* Membranes were incubated at pH 6.4 with DIDS (1 mM) in the presence or absence of 100 mM NaCl for 30 min, then diluted in 30 vol. of 50 mM HEPES (pH 7.4), and collected by centrifugation at 45,650 *g* for 20 min. This washing step was repeated once to ensure removal of the chloride (which can act as a competitive inhibitor of the binding of metolazone [19]). Finally, the membrane pellets were resuspended in 50 mM HEPES buffer (pH 7.4) for the [^{3}H]metolazone binding assay. Control membranes were treated identically except that the DIDS was omitted. The subsequently determined binding of [^{3}H]metolazone was irreversibly inhibited 52.0 ± 2.22% in membranes treated with DIDS alone and 72.0 ± 3.27% in membranes treated with DIDS in the presence of 100 mM NaCl (Fig. 5). The inhibition in the presence of NaCl was significantly greater than in its absence ($P < 0.001$). Thus, instead of protecting against inhibition, chloride and sodium actually augmented the ability of DIDS to inhibit the binding of [^{3}H]-metolazone, as did Na_2SO_4 and Cs_2SO_4 (two non-halide salts that do not inhibit binding of [^{3}H]-metolazone to the thiazide receptor [19]) (Fig. 5).

DISCUSSION

Diuretics of the benzothiadiazine class and related compounds exert their pharmacological action, presumably via interaction with a specific "receptor" for this class of drugs, by blocking reabsorption of chloride and sodium in a distal portion of the renal tubule [5, 23, 24]. We have utilized [^{3}H]metolazone to detect, by classical ligand binding techniques, a site in renal membranes that binds [^{3}H]metolazone with the pharmacological specificity expected of the thiazide receptor [9]. Importantly, the binding of [^{3}H]metolazone is inhibited by chloride ion in a competitive (or mutually exclusive) fashion [19]. For example, 50 mM chloride (as the sodium salt) decreased the affinity for binding of [^{3}H]metolazone to 16% of that found in the presence of 50 mM sodium (as the sulfate salt) [19]. Thus, it appears either that chloride and metolazone occupy the same (or overlapping) sites on the thiazide receptor or that occupancy of the anion site by chloride causes a conformational change in the receptor such that it is no longer able to recognize metolazone with high affinity.

At issue in the present study was whether the anion binding site of the thiazide receptor could be inhibited by reaction with disulfonic stilbenes in a fashion analogous to the ability of disulfonic stilbenes to react with and inhibit the anion transporter in erythrocytes [13, 14] and the sulfate transporter in renal membranes [15]. If such were the case, reaction of disulfonic stilbenes with the anion site on the thiazide receptor would be expected to inhibit binding of [^{3}H]metolazone. Moreover, such a postulated identity of the sites for binding of chloride and reaction with stilbenes would mean that chloride would compete with the stilbene and delay the rate at which the stilbene could inhibit the binding of [^{3}H]metolazone. Thus, comparing the reaction of

renal membranes with the stilbene disulfonates in the absence and presence of chloride held the potential, if the reaction were irreversible, to be a method for identification of the chloride binding site on the thiazide receptor. Therefore, we conducted experiments both to characterize the reversibility of the inhibition of binding of [^{3}H]metolazone produced by disulfonic stilbenes and to determine if the inhibition was protectable by the chloride anion.

We found that micromolar concentrations of both DIDS and SITS slightly inhibited [^{3}H]metolazone binding to renal membranes, as indicated in Fig. 1. Higher concentrations (from 100 μM to 2 mM) of both agents reduced the binding dramatically, with apparent IC_{50} values of 380 μM for DIDS and 940 μM for SITS.

Irreversibility of the inhibition of binding of [^{3}H]metolazone by DIDS and SITS is supported by the results of two types of experiments. First, reaction of membranes with 1 mM DIDS or SITS at pH 6.4 for 30 min prior to removal of the DIDS by dilution and washing of the membranes with buffer did not eliminate the inhibition of binding of [^{3}H]metolazone during a subsequent 4-hr equilibration with [^{3}H]metolazone. Moreover, reaction with DIDS or SITS at pH 6.4 produced slightly more inhibition than did reaction with the respective reagents at pH 7.4 or 8.4 (Fig. 3). The isothiocyanate group of the stilbenes reacts preferentially with protonated amino (predominantly lysine) residues on membrane proteins [25]. Hence, increasing pH would result in conversion of $-NH_3^+$ to $-NH_2$ and less irreversible inhibition. Thus, our finding of lesser inhibition at pH 7.4 and pH 8.4 than at pH 6.4 (Figs. 3 and 4) is consistent with preferential reaction of the reagents with one or more protonated amino groups on the thiazide receptor. Second, inclusion of 1% BSA in the washing buffer and additional washing did not reduce the inhibition markedly (Figs. 3 and 4). (BSA has been used to react with residual stilbenes and to strip non-covalently bound stilbenes from membrane proteins [15].) Therefore, the inhibition of binding of [^{3}H]metolazone produced by 1 mM DIDS and SITS in our study has the properties expected of an irreversible inhibition due to covalent binding to amino groups on the thiazide receptor.

We note that incubation of membranes with 1 mM SITS, pH 6.4, decreased binding of [^{3}H]metolazone by 27.0 ± 3.69% even when 1% BSA was included in the wash solutions (Fig. 4). This degree of inhibition is less than the 52.4 ± 3.24% inhibition produced under the same condition by DIDS (Fig. 3). The lesser inhibition by SITS may be attributable to the structural difference between DIDS and SITS in that the latter has only one isothiocyanate group that can form a covalent bond.

As mentioned previously, if DIDS inhibits by reacting at the site on the thiazide receptor which binds chloride, inclusion of chloride in the reaction mixture would be expected to protect the site binding [^{3}H]metolazone from inhibition by DIDS. However, our data (Fig. 5) indicate that chloride did not protect the thiazide receptor from inhibition by DIDS. Thus, we infer that DIDS reduces the number of sites able to bind [^{3}H]metolazone (Table 1) by reacting at a site other than the anion/[^{3}H]metolazone binding site on the thiazide receptor. In fact, the increased extent of inhibition produced by DIDS in the presence of NaCl appears to represent a nonspecific effect of ionic strength, since the sulfate salts of both sodium and cesium increased the extent of inhibition of binding of [^{3}H]metolazone produced by DIDS (Fig. 5) as effectively as did the chloride salt of sodium. In this regard, it is important to note that, while sodium increased and chloride decreased the affinity of binding of [^{3}H]metolazone, neither cesium nor sulfate alters the binding of [^{3}H]metolazone by renal membranes [19].

In summary, both DIDS and SITS inhibited binding of [^{3}H]metolazone to rat kidney membranes in an apparently irreversible fashion and with apparent IC_{50} values of 380 μM and 940 μM, respectively. These findings suggest that one or more amino moieties on the thiazide receptor are essential for the binding of [^{3}H]metolazone by the thiazide receptor. However, we infer that the reactive amines are located at other than in the anion binding/transporting site, since NaCl did not protect against the inhibition of binding. Moreover, disulfonic stilbenes appear not to be ideal specific markers for future identification of the thiazide receptor, since relatively high concentrations of DIDS or SITS were required to inhibit binding of [^{3}H]metolazone.

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REFERENCES

1. Kunau RT, Weller DR and Webb HL, Clarification of the site of action of chlorothiazide in the rat nephron. *J Clin Invest* **56**: 401–407, 1975.
2. Costanzo LS and Windhager EE, Calcium and sodium transport by the distal convoluted tubule of the rat. *Am J Physiol* **246**: F492–F506, 1978.
3. Costanzo LS, Localization of diuretic action in microperfused rat distal tubules: Ca and Na transport. *Am J Physiol* **248**: F527–F535, 1985.
4. Ellison DH, Velazquez H and Wright FS, Thiazide-sensitive sodium chloride cotransport in the early distal tubule. *Am J Physiol* **253**: F546–F554, 1987.
5. Velazquez H, Good DW and Wright FS, Mutual dependence of sodium and chloride absorption by renal distal tubule. *Am J Physiol* **247**: F904–F911, 1984.
6. Suki WN, Dawoud F, Eknoyan G and Martinez-Maldonado M, Effects of metolazone on renal function in normal man. *J Pharmacol Exp Ther* **180**: 6–12, 1972.
7. Steinmullar SR and Puschett JB, Effects of metolazone in man: Comparison with chlorothiazide. *Kidney Int* **1**: 169–181, 1972.
8. Shetty BV, Campanella LA, Thomas TL, Fedorchuk M, Davidson TA, Michelson L, Volz H, Zimmerman SE, Belair EJ and Truant AP, Synthesis and activity of some 3-aryl- and 3-aralkyl-1,2,3,4-tetrahydro-4-oxo-6-quinazolinesulfonamides. *J Med Chem* **13**: 886–895, 1970.
9. Beaumont K, Vaughn DA and Fanestil DD, Thiazide diuretic drug receptors in rat kidney: Identification with [^{3}H]metolazone. *Proc Natl Acad Sci USA* **85**: 2311–2314, 1988.
10. Beaumont K, Vaughn DA and Healy DP, Thiazide

diuretic receptors: Autoradiographic localization in rat kidney with [^{3}H]metolazone. *J Pharmacol Exp Ther* **250**: 414–419, 1989.
11. Pearce SFA and Zadunaisky JA, Fluorescent stilbene (BADS) binding proteins in anion-transporting epithelia. *Am J Physiol* **259**: C439–C449, 1990.
12. Maddy H, A fluorescent label for the outer components of the plasma membrane. *Biochim Biophys Acta* **88**: 390–399, 1964.
13. Cabantchik ZI and Rothstein A, The nature of the membrane sites controlling anion permeability of human red blood cells as determined by studies with disulfonic stilbene derivatives. *J Membr Biol* **10**: 311–330, 1972.
14. Lepke S, Fasold H, Pring M and Passow H, A study of the relationship between inhibition of anion exchange and binding to the red blood cell membrane of 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and of its dihydro derivative (H_2DIDS). *J Membr Biol* **29**: 147–177, 1976.
15. Bästlein C and Burckhardt G, Sensitivity of rat renal luminal and contraluminal sulfate transport systems to DIDS. *Am J Physiol* **250**: F226–F234, 1986.
16. Beaumont K and Vaughn DA, Renal thiazide diuretic receptors: Site heterogeneity. *Proc West Pharmacol Soc* **31**: 259–261, 1988.
17. Beaumont K, Vaughn DA, Maciejewski AR and Fanestil DD, Reversible downregulation of thiazide diuretic receptors by acute renal ischemia. *Am J Physiol* **256**: F329–F334, 1989.
18. Beaumont K, Vaughn DA, Casto R, Printz MP and Fanestil DD, Thiazide diuretic receptors in spontaneously hypertensive rats and 2-kidney 1-clip hypertensive rats. *Clin Exp Hypertens* [*A*] **12**: 215–226, 1990.
19. Tran JM, Farrell MA and Fanestil DD, Effect of ions on the binding of the thiazide-type diuretic metolazone to kidney membranes. *Am J Physiol* **258**: F908–F915, 1990.
20. Chen Z, Vaughn DA, Beaumont K and Fanestil DD, Effects of diuretic treatment and of dietary sodium on renal binding of ^{3}H-metolazone. *J Am Soc Nephrol* **1**: 91–98, 1990.
21. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
22. McPherson GA, A practical computer-based approach to the analysis of radioligand binding experiments. *Comput Programs Biomed Res* **17**: 107–114, 1983.
23. Ellison DH, Velazquez H and Wright FS, Mechanisms of sodium, potassium and chloride transport by the renal distal tubule. *Min Electrolyte Metab* **13**: 422–432, 1987.
24. Stokes JB, Sodium chloride absorption by the urinary bladder of the winter flounder. A thiazide-sensitive, electrically neutral transport system. *J Clin Invest* **74**: 7–16, 1984.
25. Cabantchik ZI, Knauf PA and Rothstein A, The anion transport system of the red blood cell. The role of membrane protein evaluated by the use of probes. *Biochim Biophys Acta* **515**: 239–302, 1978.