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J Clin Invest. 1988;82(2):721-730. <https://doi.org/10.1172/JCI113653>.

Research Article

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Site and Mechanism of Action of Trichlormethiazide in Rabbit Distal Nephron Segments Perfused In Vitro

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Abstract

To determine the exact site and mechanism of action of thiazide diuretics, effects of 10^{-4} M trichlormethiazide (TCM) on NaCl transport were examined in the distal convoluted tubule (DCT), the connecting tubule (CNT) and the cortical collecting duct (CCD) of rabbit kidney by the in vitro microperfusion technique. TCM added to the lumen decreased lumen-to-bath ^{36}Cl flux ($J_{\text{Cl(LB)}}$) only in the CNT without changing the transmural voltage (V_T). In the DCT, 10^{-4} M furosemide did not change $J_{\text{Cl(LB)}}$ even if it was added to the lumen with 10^{-4} M TCM, whereas 10^{-5} M amiloride in the lumen decreased the lumen-to-bath ^{22}Na flux ($J_{\text{Na(LB)}}$) and V_T . In the CNT, TCM added to the lumen did not affect the bath-to-lumen ^{36}Cl flux. Addition of TCM to the bath slightly decreased $J_{\text{Cl(LB)}}$. Luminal addition of 10^{-4} M TCM also decreased $J_{\text{Na(LB)}}$. Amiloride at 10^{-5} M in the lumen decreased both $J_{\text{Na(LB)}}$ and V_T . Addition of TCM with 10^{-5} M amiloride further decreased $J_{\text{Na(LB)}}$ without affecting V_T , indicating that TCM affects the electroneutral Na^+ transport, which is distinct from the amiloride-sensitive conductive Na^+ pathway. When Na^+ was removed from the lumen, $J_{\text{Cl(LB)}}$ was markedly decreased, but addition of TCM did not cause further decrease in $J_{\text{Cl(LB)}}$. Furosemide did not affect $J_{\text{Cl(LB)}}$, but addition of both 10^{-4} M TCM and furosemide decreased $J_{\text{Cl(LB)}}$, indicating that $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransport is not involved in the action of TCM. Removal of HCO_3^- slightly decreased $J_{\text{Cl(LB)}}$, and TCM caused further decrease in $J_{\text{Cl(LB)}}$. Amiloride at 10^{-3} M, a concentration supposed to inhibit the Na^+/H^+ antiport, slightly decreased $J_{\text{Cl(LB)}}$, and addition of TCM caused a further marked decrease in $J_{\text{Cl(LB)}}$. The similar results were also obtained when the combined effects of 10^{-3} M 4,4'-diisothiocyano-stilben-2,2'-disulfonate (DIDS) and 10^{-4} M TCM were examined. These findings suggest that the parallel antiport of Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ is not involved in the action of TCM. By excluding other possible mechanisms involving neutral Na^+ -dependent Cl^- transport, we conclude that TCM inhibits Na^+-Cl^- cotransport in the luminal membrane of the rabbit CNT.

Introduction

Although thiazide diuretics have been widely used in the treatment of edema and hypertension for many years, the exact

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Received for publication 27 October 1987 and in revised form 17 March 1988.

J. Clin. Invest.

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0021-9738/88/08/0721/10 \$2.00

Volume 82, August 1988, 721-730

sites and mechanisms of action within the kidney are not definitely established. The observations that thiazide diuretics decreased free water clearance without affecting free water reabsorption provided indirect evidence that they act on the so-called cortical diluting segments (1-3), which include the cortical thick ascending limb of Henle's loop as well as other distal nephron segments.

More direct evidence for distal nephron site of action of thiazides was provided by the in vivo micropuncture and microperfusion studies (4-10). However, it is well known that the distal convoluted tubule as defined by the micropuncture technique contains structurally as well as functionally distinct three nephron segments (11-14) including the distal convoluted tubule (DCT),¹ the connecting tubule (CNT), and the cortical collecting duct (CCD). In vivo microperfusion studies in rat distal nephron (8, 10) demonstrated that thiazide diuretics act only on early portion of the distal nephron, suggesting that the distal convoluted tubule is the site of action of thiazide diuretics. In the rabbit, the transitions from one to the other segment along the distal nephron are abrupt (11), and therefore functions of a well defined segment can be examined by the in vitro microperfusion technique (13-17).

This study elucidates the site and mechanism of action of thiazide diuretics in well defined distal nephron segments. For this purpose, we examined effects of trichlormethiazide (TCM) on NaCl transport in the rabbit distal nephron segments by the in vitro microperfusion technique. We report that TCM inhibits Na^+-Cl^- cotransport by acting exclusively on the CNT, at least in the rabbit kidney.

Methods

In vitro microperfusion of isolated renal tubules. The technique of isolated renal tubular perfusion developed by Burg et al. (18) was employed as modified previously (13, 15). Male Japanese white rabbits, maintained on standard rabbit chow and tap water, were anesthetized with pentobarbital (35 mg/kg, i.v.) and kidneys were removed. Kidney slices 1-3 mm thickness were made and placed in a cooled dish containing an artificial solution simulating intracellular ion composition (KCl 14, K_2HPO_4 , 44, KH_2PO_4 14, NaHCO_3 9, sucrose 160 mM; pH 7.4). The composition was same as Collin's solution except that glucose was replaced by sucrose. This dissection medium was selected because it has been reported that intracellular fluid-like solutions are much better in preserving kidney tissue metabolically (19) as well as functionally (20, 21). We have also confirmed in preliminary studies that the function of rabbit proximal straight tubule was well preserved even after kidney slices were kept at 5°C for 24 h.

Three different distal nephron segments, including the DCT, CNT,

1. Abbreviations used in this paper: CCD, cortical collecting duct; CNT, connecting tubule; DCT, distal convoluted tubule; DIDS, 4,4'-diisothiocyano-stilben-2,2'-disulfonate; $J_{\text{Cl(BL)}}$, bath-to-lumen Cl^- flux; $J_{\text{Cl(LB)}}$, lumen-to-bath Cl^- flux; $J_{\text{Na(LB)}}$, lumen-to-bath Na^+ flux; TCM trichlormethiazide; V_T , transmural voltage.

and CCD, were isolated by identifying them according to the following criteria. The DCT was obtained from the superficial nephron. The segment was located in the vicinity of the superficial glomerulus, and was identified by its bright appearance. The CNT was obtained from the nephron arcade, and was identified by the existence of at least two branches and its granular appearance. The CCD was obtained from the medullary ray and was identified by its straight shape and light appearance. The validity of this criteria was confirmed in each instance by morphological appearance of epithelia observed on an inverted microscope during perfusion of the tubules. Detailed morphological descriptions and photographs of these segments have been reported previously (13).

The isolated tubules were transferred to a temperature controlled bath and were perfused at 37°C. The compositions of artificial solutions used in this study are shown in Table I. Bicarbonate Krebs Ringer (BKR) solution was the main perfusion medium. In some experiments, the sodium in the lumen was replaced with choline, or the bicarbonate in the bath and perfusate was replaced with Hepes-glucuronate (Table I). Bicarbonate-containing solution was bubbled with 95% O₂-5% CO₂ to attain a pH of 7.4. Bicarbonate-free solution was bubbled with 100% O₂.

The electrical circuit to measure the transmural voltage (V_T) was identical to that previously reported (13). A calomel half-cell electrode was connected by an agar bridge to the perfusion pipette. Another electrode was connected by an agar bridge to the bath. The electrical potential difference between the two electrodes was measured with an electrometer (Keithly 602, Keithly Instruments, Inc., Cleveland, OH). It has been reported that the V_T of the CNT and DCT were always negative in the lumen and were pressure-dependent (13, 22). In the CNT, when the perfusion pressure was varied from 2 to 40 cm H₂O, the V_T decreased as a function of the height of reservoir from about -20 to -2 mV. Therefore, this study was performed at a fixed height of the fluid reservoir connected to the perfusion pipette of ~ 10 cm H₂O. Under these experimental conditions, the perfusion rate and V_T were maintained between 5 and 10 nl min⁻¹ and between 0 and -10 mV, respectively.

Unidirectional fluxes of ³⁶Cl or ²²Na from lumen to bath ($J_{Cl(LB)}$ or $J_{Na(LB)}$) were measured by adding 3 μCi/ml ³⁶Cl or 10 μCi/ml ²²Na to the perfusate. The CCD is virtually impermeable to water in the absence of vasopressin, and both DCT and CNT are impermeable to water even in the presence of vasopressin (13, 22). Therefore, $J_{Cl(LB)}$ or $J_{Na(LB)}$ can be calculated using the equation; $J_{Cl(LB)} = (V_0[C]_i/L)(1 - [C^*]_0/[C^*]_i)$, where $[C]_i$ is the concentration of solute in the perfusate, V_0 is the collection rate, L is the tubular length, and $[C^*]_i$ and $[C^*]_0$ are concentrations of isotope in the perfusate and the collected fluid, respectively. The lumen-to-bath flux coefficient of Cl⁻

($K_{Cl(LB)}$) or Na⁺ ($K_{Na(LB)}$) was calculated as follows (23): $K_{Cl(LB)} = (V_0/L) \ln([C^*]_i/[C^*]_0)$. The bath-to-lumen flux coefficient ($K_{Cl(BL)}$) and the influx of ³⁶Cl from bath to lumen ($J_{Cl(BL)}$) were calculated as (24); $K_{Cl(BL)} = (V_0/L) \ln\{[C^*]_b/([C^*]_b - [C^*]_0)\}$ and $J_{Cl(BL)} = [Cl]_b K_{Cl(BL)}$, where $[Cl]_b$ is the concentration of Cl⁻ in the bath. $[C^*]_0$ and $[C^*]_i$ are the concentrations of ³⁶Cl in the collected fluid and the bath, respectively.

Net Cl⁻ flux ($J_{Cl(net)}$) was measured by adding ³⁶Cl both to the perfusate and the bathing fluid at same concentration. $J_{Cl(net)}$ was calculated as; $J_{Cl(net)} = (V_0[Cl]_i/L)(1 - [C^*]_0/[C^*]_i)$, where $[Cl]_i$ is Cl⁻ concentration in the perfusate, V_0 is the collection rate, L is the tubular length, $[C^*]_0$ and $[C^*]_i$ are concentration of ³⁶Cl in the collected fluid and perfusate, respectively.

Usually, we used data of unidirectional fluxes to represent transport parameters, but we also gave flux coefficients in tables for convenience of comparison of fluxes normalized by ion concentration.

The radioactivity of ³⁶Cl was measured with a liquid scintillation counter (LKB Wallac, 1217 Rack Beta; LKB Instruments, Gaithersburg, MD). Radioactivity of ²²Na was measured with a gamma counter (LKB Wallac, 1282 Compu Gamma).

Statistical analysis. The mean value for three collection periods was used as a representative value for a given experimental condition. Data were expressed as mean ± SE. Statistical analysis was performed by either the paired or nonpaired *t* test, when appropriate.

Chemicals. TCM (Shionogi Co. Ltd., Osaka, Japan) was obtained as pure compound and dissolved in dimethylsulfoxide (DMSO) immediately before use. The original solutions were diluted at least 100-fold with an artificial solution used for the perfusion experiments to obtain the final concentration of 10⁻⁴ M. In preliminary studies we confirmed that this amount of DMSO had no effect on net volume flux, lumen-to-bath Cl⁻ flux and V_T in all segments used in this study (Table II). Amiloride and furosemide were purchased from Sigma Chemical Co., (St. Louis, MO) and Hoechst Japan (Tokyo), respectively, 4,4'-diisothiocyanato-stilben-2,2'-disulfonate (DIDS) was obtained from Funakoshi Chemical Co. (Tokyo). ²²Na and ³⁶Cl were products of New England Nuclear (Boston, MA) and Amersham International (Buckinghamshire, UK), respectively.

Results

Site of action of TCM in the distal nephron segments. To clarify the site of action of TCM in the distal nephron segments, we observed the effect of TCM on the lumen-to-bath Cl⁻ flux in the DCT, CNT and CCD. The results are summarized in Table III and individual data are depicted in Fig. 1. When 10⁻⁴ M TCM was added to the lumen, Cl⁻ flux was reduced only in the CNT but not in the DCT and CCD. In the CNT, $J_{Cl(LB)}$ was decreased from 914 to 657 pmol mm⁻¹ min⁻¹ and $K_{Cl(LB)}$ from 17.12 to 11.25 × 10⁻⁷ cm² s⁻¹ without significant change in V_T . Thus the CNT is the target of the action of TCM. Since the data obtained by in vivo microperfusion studies in rats (8, 10) strongly suggest that the DCT is the site of action of thiazide diuretics, we have carefully conducted 2 additional protocols to characterize the segments of DCT more specifically. In the first protocol, we observed effects of furosemide and TCM on the lumen-to-bath Cl⁻ flux in the DCT. The results are summarized in Fig. 2. Administration of 10⁻⁴ M furosemide in the lumen did not affect the Cl⁻ flux or the transmural voltage. Addition of TCM 10⁻⁴ M to the lumen in combination with furosemide did not change these parameters as well. These observations indicate that the segment defined as the DCT is clearly distinct from the thick ascending limb, which is supposed to display lumen positive voltage and where Cl⁻ transport is inhibited by furosemide. In this series of experiments, we confirmed again that TCM was without effect on Cl⁻ flux in the DCT.

Table I. Composition of Perfusing and Bathing Solutions

Solution	BKR	Na ⁺ -free	Bicarbonate-free
Na ⁺	140.5	—	140.5
K ⁺	5.0	5.0	5.0
Ca ²⁺	1.8	1.8	1.8
Mg ²⁺	1.0	1.0	1.0
Choline	—	138.3	—
Cl ⁻	120.6	120.6	120.6
HCO ₃ ⁻	25.0	25.0	—
HPO ₄ ²⁻ /H ₂ PO ₄ ⁻	1.0	1.0	1.0
Glucuronate	—	—	15.0
Hepes	—	—	10.0

Concentrations are shown in mM. All solutions contain in mM: D-glucose 3.8, L-alanine 5.0, citrate 1.5, and glutamate 3.5. BKR, bicarbonate Krebs-Ringer.

Table II. Effect of 1% Dimethylsulfoxide on Net Water Flux (J_v), $J_{Cl(LB)}$, and V_T in the DCT, CNT, and CCD

Segments	(n)	DMSO	V_0	J_v	$J_{Cl(LB)}$	V_T
		%	nl min ⁻¹	nl mm ⁻¹ min ⁻¹	pmol mm ⁻¹ min ⁻¹	mV
DCT	(4)	0	8.73±0.77	-0.04±0.05	444±62	-3.9±1.5
		1	7.97±0.69	0.04±0.18	404±12	-4.0±1.6
CNT	(5)	0	8.61±0.79	-0.03±0.12	614±49	-8.1±1.1
		1	8.37±0.55	0.06±0.08	661±118	-8.2±1.0
CCD	(5)	0	8.63±0.54	0.07±0.09	252±51	-6.7±2.3
		1	9.04±0.65	0.08±0.04	242±42	-6.8±2.2

n = number of tubules.

Gross et al. (22) have reported that administration of amiloride to the lumen of the rabbit DCT suppressed the lumen negative V_T . Therefore, in the second protocol, we intended to confirm and extend their observation by examining effects of amiloride on the lumen-to-bath Na^+ flux. As summarized in Fig. 3, 10^{-5} M amiloride added to the lumen decreased the V_T as well as Na^+ flux. These effects were reversible.

All the following studies are confined to the CNT. Seven separate experiments were conducted to examine the effect of luminal application of TCM on the bath-to-lumen Cl^- flux (Table IV). The results show that TCM did not affect the bath-to-lumen Cl^- flux. However, it is difficult to estimate the net Cl^- flux across the CNT by comparing with the data shown in Table III, since bidirectional Cl^- fluxes were not measured in the same tubule. Because of large scatter of the data, we are not allowed to compare the unpaired samples without doing a large scale of experiments. In order to examine whether TCM decreases net Cl^- flux, we measured net Cl^- flux under the condition where ^{36}Cl concentration of the perfusing fluid was identical to that in the bathing fluid. The results are summarized in Table V. It is clear that TCM decreases net Cl^- flux in the CNT.

In order to examine whether the luminal application is the major route of diuretic action, we observed the effect of TCM

added to the bath and or lumen on $J_{Cl(LB)}$. After the control period, 10^{-4} M TCM was added at first to the bath, and then the drug was added to the lumen with the drug concentration in the bath being kept constant. In the last period, the drug was eliminated only from the lumen. The results are summarized in Fig. 4. When TCM was added to the bath a small but significant decrease in $J_{Cl(LB)}$ was observed. However, addition of 10^{-4} M TCM to the lumen in the presence of the drug in the bath caused a much greater reduction of $J_{Cl(LB)}$ from 818 to 551 pmol mm⁻¹ min⁻¹.

Effect on Na^+ flux. To examine whether the inhibition of Cl^- transport by TCM is associated with an inhibition of Na^+ transport, we observed effect of 10^{-4} M TCM in the lumen on the lumen-to-bath ^{22}Na flux. The results are summarized in Table VI. The lumen-to-earth ^{22}Na flux in the control period was lower than the value for Cl^- . Administration of 10^{-4} M TCM to the lumen caused a marked decrease in $J_{Na(LB)}$ from 523 to 354 pmol mm⁻¹ min⁻¹. This effect of TCM was reversible. These data indicate that TCM inhibits Na^+ flux as well as Cl^- flux.

Since TCM inhibited NaCl transport in the CNT without affecting V_T , it is unlikely that the drug influences rheogenic transport processes. To confirm this issue, we examined whether TCM inhibits only the Na^+ flux which is independent

Table III. Effect of 10^{-4} M TCM in the Lumen on Cl^- Transport in the Distal Nephron Segments

Period	Lumen TCM	V_0	$K_{Cl(LB)}$	$J_{Cl(LB)}$	T_v
	M	nl/min	$10^{-7} cm^2 s^{-1}$	pmol mm ⁻¹ min ⁻¹	mV
<i>DCT (n = 7, L = 0.42±0.05 mm)</i>					
C	0	5.75±0.37	9.04±1.16	528±88	-6.8±1.7
E	10^{-4}	5.18±0.15	8.47±1.67	510±92	-6.6±1.9
R	0	5.23±0.29	8.81±1.77	534±94	-5.6±1.6
<i>CNT (n = 8, L = 0.34±0.05 mm)</i>					
C	0	5.04±0.25	17.12±2.54	914±123	-4.8±1.4
E	10^{-4}	5.73±0.58	11.25±1.75*	657±94*	-4.6±1.6
R	0	5.74±0.73	13.93±2.16*	765±127*	-4.8±1.5
<i>CCD (n = 4, L = 0.77±0.05 mm)</i>					
C	0	7.17±0.92	12.52±2.12	627±105	-4.3±1.7
E	10^{-4}	7.50±0.29	11.54±2.54	604±113	-4.4±1.6
R	0	7.61±0.51	10.63±2.19	563±100	-3.8±1.3

Abbreviations used in this table: C, control period; E, experimental period; R, recovery period. $K_{Cl(LB)}$, lumen-to-bath flux coefficient for Cl^- ; L, tubular length; V_0 , perfusion rate; * $P < 0.01$.

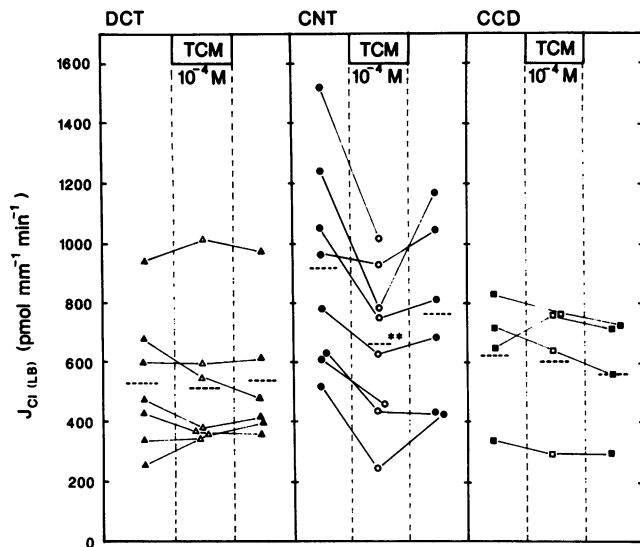


Figure 1. Effects of TCM on $J_{Cl(LB)}$ in distal nephron segments. Trichlormethiazide was added to the lumen. * $P < 0.01$ as compared to the preceding values.

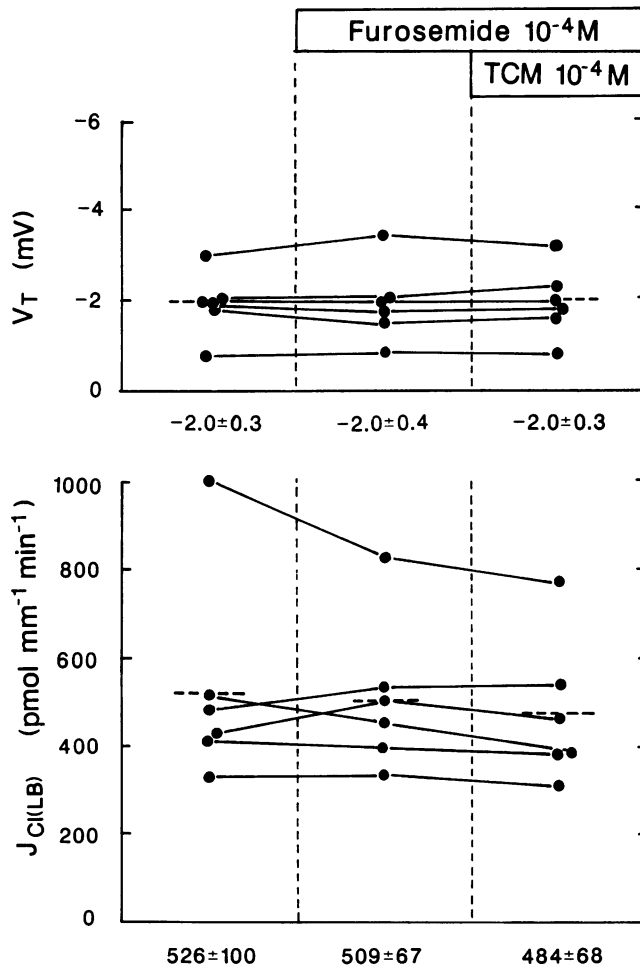


Figure 2. Effects of 10^{-4} M furosemide and 10^{-4} M TCM on V_T and $J_{Cl(LB)}$ in the DCT. Tubular length was 0.38 ± 0.03 mm. Perfusion rates for each period were 8.24 ± 1.69 , 7.92 ± 1.15 , and 7.48 ± 1.11 nl/min, respectively.

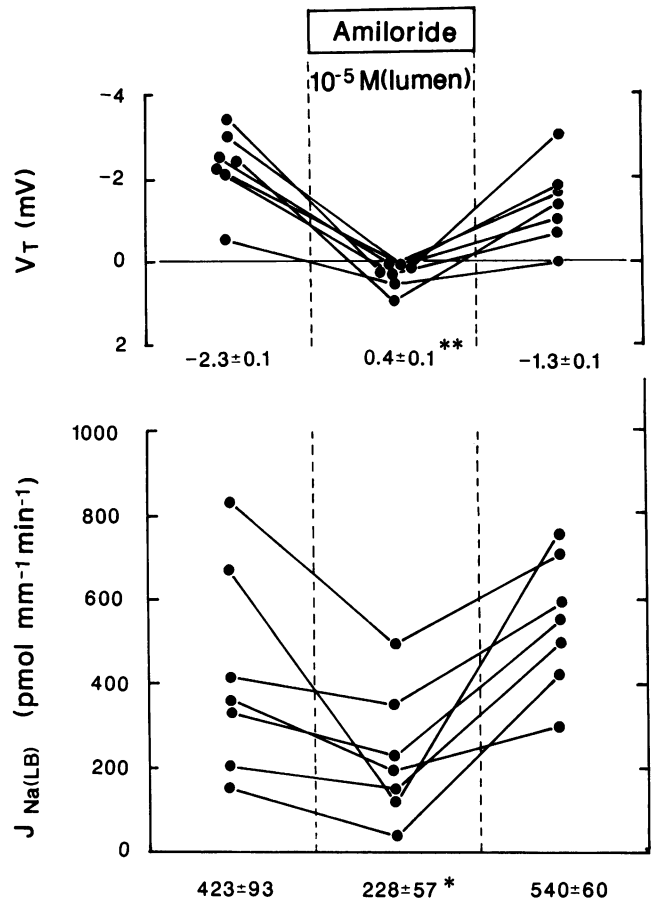


Figure 3. Effects of 10^{-5} M amiloride in the lumen on V_T and lumen-to-bath Cl^- flux $J_{Cl(LB)}$ in the DCT. Values are means \pm SE. Tubular length was 0.41 ± 0.02 . ** $P < 0.01$, * $P < 0.05$ as compared to the preceding values.

of the amiloride sensitive component. After the control period, 10^{-5} M amiloride was added to the lumen. In the next period, 10^{-4} M TCM was added to the lumen in the presence of 10^{-5} M amiloride. In the final period, recovery from the effects of these drugs was observed. The results of this protocol are summarized in Fig. 5. Amiloride added to the lumen at 10^{-5} M inhibited Na^+ flux from 649 to 426 $pmol\ mm^{-1}\ min^{-1}$ in association with a significant reduction in V_T from -2.0 to -0.1 mV. Under this circumstance, administration of 10^{-4} M TCM in the lumen further decreased the Na^+ flux to 364 $pmol\ mm^{-1}\ min^{-1}$ without causing changes in V_T . The parameter tended to recover when the drugs were eliminated from the lumen. These observations clearly indicate that there is an

Table IV. Effect of 10^{-4} M TCM in the Lumen on Bath-to-Lumen ^{36}Cl -flux in the CNT

Period	V_0 nl min ⁻¹	$K_{Cl(BL)}$ $10^{-7}\ cm^2\ s^{-1}$	$J_{Cl(BL)}$ pmol mm ⁻¹ min ⁻¹	V_T mV
Control	9.77 ± 0.55	11.92 ± 1.37	862 ± 99	-3.7 ± 0.8
TCM 10^{-4}	9.05 ± 0.14	12.50 ± 1.36	904 ± 99	-3.5 ± 0.9

Data are from seven tubules with length of 0.39 ± 0.02 mm. $K_{Cl(BL)}$, bath-to-lumen flux coefficient for Cl^- .

Table V. Effect of 10^{-4} M TCM in the Lumen on Net Cl^- Flux in the CNT

Period	TCM (lumen)	V_0	$J_{\text{Cl}(\text{net})}$	V_T
		nl/min	pmol $\text{mm}^{-1} \text{min}^{-1}$	mV
C	0	9.50±0.69	405±55	-2.2±0.5
E	10^{-4} M	8.83±0.66	281±59*	-2.1±0.5
E-C		0.67±0.04	$\Delta-124\pm48^*$	$\Delta+0.1\pm0.2$

Data are from seven tubules with length of 0.38 ± 0.03 mm. Net Cl^- flux, $J_{\text{Cl}(\text{net})}$, was measured by adding ^{36}Cl to both perfusing and bathing fluid at the same concentration.

* $P < 0.05$.

amiloride-sensitive Na^+ transport process in the CNT but that TCM inhibits the Na^+ flux, which is independent of amiloride action.

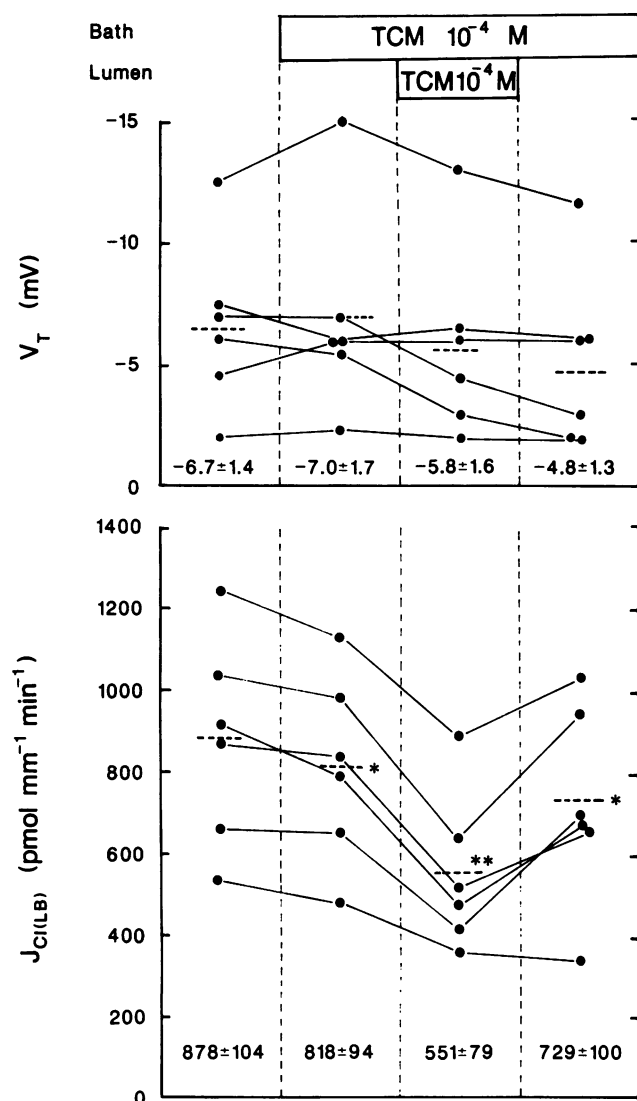


Figure 4. Examination of sidedness of the action of TCM in the CNT. Values are means±SE. Tubular length was 0.37 ± 0.05 mm. Perfusion rates in four periods were 6.28 ± 0.39 , 6.15 ± 0.36 , 5.74 ± 0.30 , and 6.29 ± 0.24 nl/min, respectively. * $P < 0.05$, ** $P < 0.01$ as compared to the preceding values.

Table VI. Effect of 10^{-4} M TCM in the Lumen on Na^+ Transport in the CNT

Period	Lumen TCM	V_0	$K_{\text{Na}(\text{LB})}$	$J_{\text{Na}(\text{LB})}$	V_T
		nl min^{-1}	$10^{-7} \text{cm}^2 \text{s}^{-1}$	pmol $\text{mm}^{-1} \text{min}^{-1}$	mV
C	0	8.24±0.50	6.84±0.65	523±49	-1.9±0.9
E	10^{-4}	8.23±0.59	4.47±0.54*	354±42*	-2.0±0.1
R	0	7.85±0.56	6.64±0.63*	504±47*	-2.1±0.8

Data are from eight tubules length of 0.39 ± 0.03 mm. $K_{\text{Na}(\text{LB})}$, lumen-to-bath flux coefficient for Na^+ ; * $P < 0.01$ as compared to the values in the preceding periods.

Na^+ dependence of Cl^- flux. Since TCM inhibited both Na^+ and Cl^- fluxes without affecting V_T , it is highly possible that TCM affects Na^+ dependent Cl^- transport mechanisms including $\text{Na}^+\text{-Cl}^-$ cotransport, $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransport, and $(\text{Na}^+/\text{H}^+)\text{-}(\text{Cl}^-/\text{HCO}_3^-)$ double antiport. Therefore, we first examined whether there are Na^+ -dependent Cl^- transport processes in the luminal membrane of the CNT by observing the effect of elimination of Na^+ from the lumen on the lumen-to-bath Cl^- flux. After the control period, Na^+ was eliminated

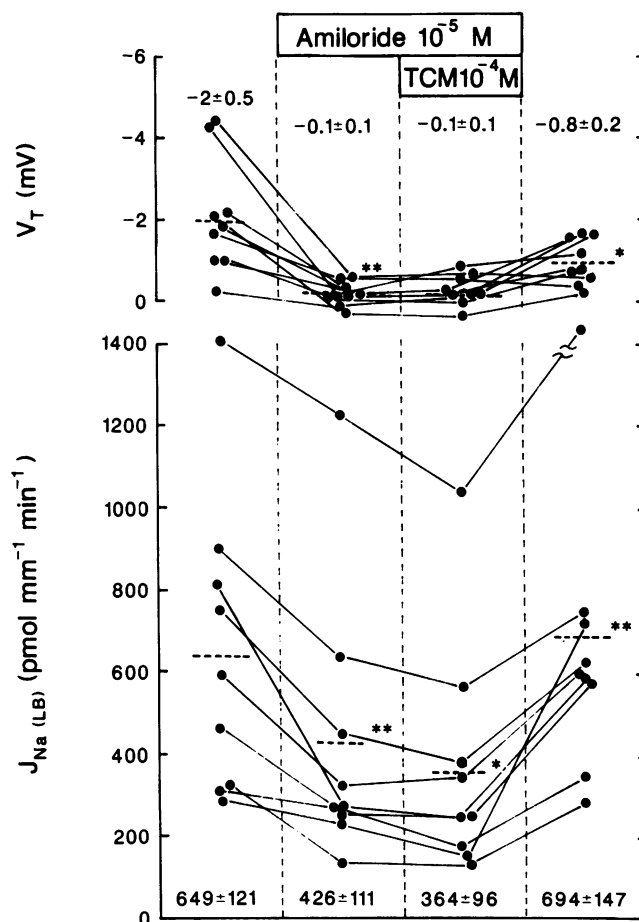


Figure 5. Effects of 10^{-5} M amiloride and 10^{-4} M TCM on V_T and $J_{\text{Na}(\text{LB})}$ in the CNT. Values are mean±SE. Tubular length was 0.42 ± 0.05 mm. Perfusion rates in four periods were 8.46 ± 0.54 , 8.10 ± 0.40 , 8.03 ± 0.53 , and 8.78 ± 0.45 nl/min, respectively. * $P < 0.05$, ** $P < 0.01$ as compared to the preceding values.

from the lumen. In the next period, 10^{-4} M TCM was added to the lumen in the absence of Na^+ . In the final period, the recovery from these maneuvers was observed. The results are summarized in Table VII. The elimination of Na^+ from the lumen decreased $J_{\text{Cl(LB)}}$ from 964 to 706 $\text{pmol mm}^{-1} \text{min}^{-1}$. In the absence of Na^+ in the lumen, addition of 10^{-4} M TCM to the lumen did not cause any significant reduction in $J_{\text{Cl(LB)}}$. These observations indicate that there is a Na^+ -dependent Cl^- transport system and that sodium is essential for inhibitory effect of TCM on chloride flux.

Effect of furosemide. In order to examine whether $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransport contributes as a target of TCM action, we examined the effect of furosemide on Cl^- flux across the CNT. The results are summarized in Fig. 6. Addition of 10^{-4} M furosemide to the lumen did not change $J_{\text{Cl(LB)}}$ as well as V_T . When 10^{-4} M TCM was added to the lumen in the presence of furosemide, $J_{\text{Cl(LB)}}$ decreased from 1111 to 569 $\text{pmol mm}^{-1} \text{min}^{-1}$ without any change in V_T . Since this concentration of furosemide is known to suppress $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransport in the thick ascending limb of Henle's loop, these observations may indicate that there is no such transport system in the CNT and that the target of TCM action is a Na^+ -dependent Cl^- transport system other than this triple cotransport.

Effect on parallel antiport of Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$. It has been reported in various epithelia that the parallel antiport of Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ is one of the mechanisms which represent apparent Na^+ -dependent Cl^- transport. In order to examine whether the bicarbonate-dependent Cl^- transport, if exists in the CNT, is the major component of the inhibitory effect of TCM, we observed effect of HCO_3^- elimination on Cl^- flux in the presence or absence of TCM. The results are summarized in Fig. 7. When HCO_3^- was eliminated from the entire system, $J_{\text{Cl(LB)}}$ decreased from 705 to 569 $\text{pmol mm}^{-1} \text{min}^{-1}$. In the absence of HCO_3^- , addition of 10^{-4} M TCM further decreased $J_{\text{Cl(LB)}}$ to 461 $\text{pmol mm}^{-1} \text{min}^{-1}$, indicating that the inhibitory effect of TCM was additive to the effect of bicarbonate elimination.

To examine whether the parallel antiport system is independent of TCM-inhibitable Cl^- flux, we observed the effect of DIDS on $J_{\text{Cl(LB)}}$ in the presence or absence of TCM. The data shown in Fig. 8 revealed that 10^{-3} M DIDS added to the lumen slightly decreased $J_{\text{Cl(LB)}}$ and that addition of 10^{-4} M TCM with 10^{-3} DIDS further caused a large decrease in $J_{\text{Cl(LB)}}$. This would indicate that the TCM-inhibitable Cl^- flux is distinct from the DIDS-inhibitable component.

Table VII. Effect of 10^{-4} M TCM in the Lumen on Cl^- Transport in the CNT under Na^+ -free Conditions

Period	Lumen	V_0	$K_{\text{Cl(LB)}}$	$J_{\text{Cl(LB)}}$
		nl min^{-1}	$10^{-7} \text{ cm}^2 \text{ s}^{-1}$	$\text{pmol mm}^{-1} \text{ min}$
C	—	7.42 ± 0.65	16.90 ± 1.74	964 ± 80
E1	Na^+ free	7.07 ± 0.57	$12.00 \pm 1.40^*$	$706 \pm 66^*$
E2	Na^+ free + 10^{-4} M TCM	6.80 ± 0.58	10.18 ± 0.93	625 ± 52
R	—	7.02 ± 0.24	$12.96 \pm 1.13^*$	$777 \pm 58^*$

Data are from seven tubules with length of 0.34 ± 0.02 mm. * $P < 0.01$ as compared to the values of preceding periods.

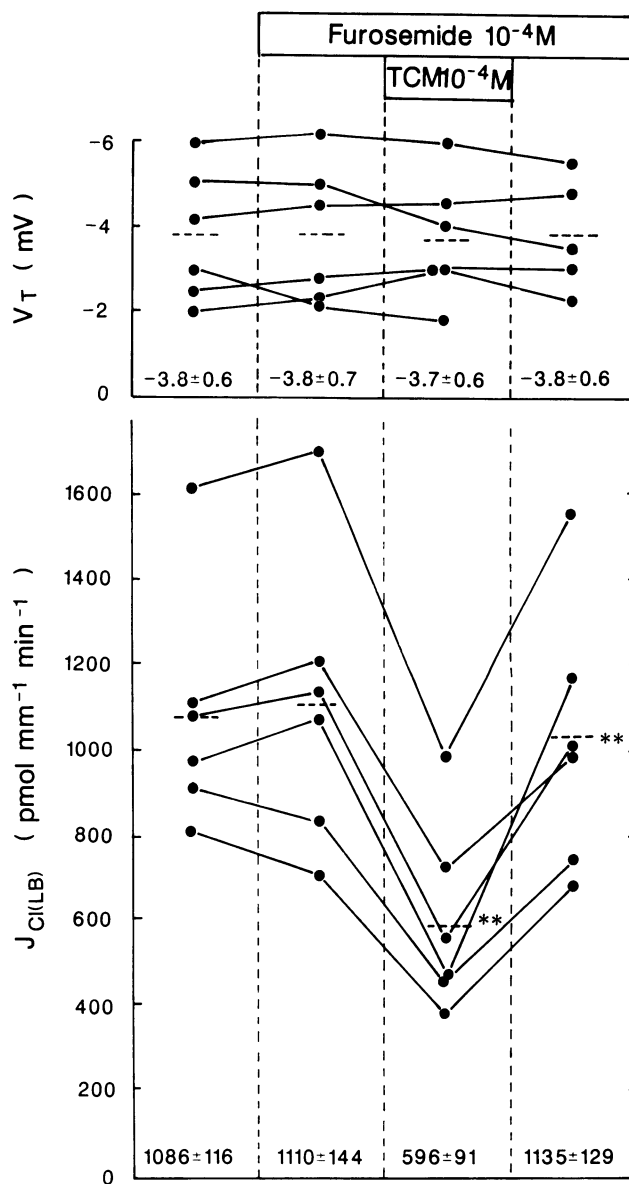


Figure 6. Effects of 10^{-4} M furosemide and 10^{-4} M TCM on V_T and $J_{\text{Cl(LB)}}$. Tubular length was 0.39 ± 0.03 mm. Perfusion rates in each period were 8.03 ± 0.37 , 8.25 ± 1.04 , 7.11 ± 0.64 , and 8.22 ± 0.42 nl/min. Values are means SE. ** $P < 0.01$ as compared with the preceding values.

To provide further evidence for this notion, we observed the effect of high concentration of amiloride on Cl^- flux. Amiloride was assumed to inhibit Na^+/H^+ antiporter at 10^{-3} M. If the parallel antiport of Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ is operating, amiloride at this concentration will cause $J_{\text{Cl(LB)}}$ to decrease. This expectation was confirmed by the experimental data shown in Fig. 9. In the presence of 10^{-3} M amiloride, 10^{-4} M TCM further decreased $J_{\text{Cl(LB)}}$. These data are also in accord with the view that the TCM-inhibitable Cl^- transport is distinct from the parallel antiport of Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$.

Discussion

Site of action of thiazide diuretics. At present time, it is generally believed that the major site of action of thiazides is the

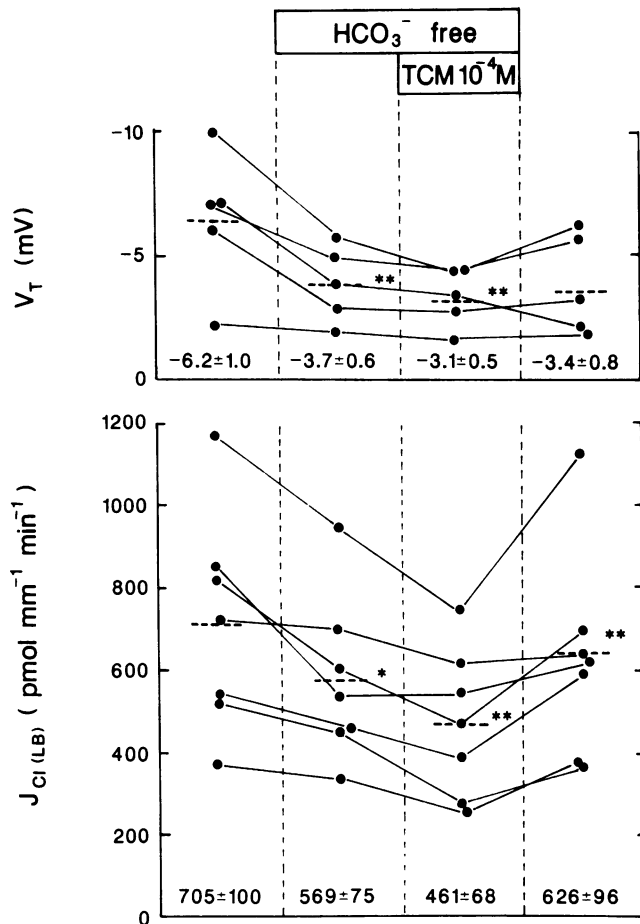


Figure 7. Effects of elimination of bicarbonate and addition of 10^{-4} M TCM on transmembrane voltage (V_T) and lumen-to-bath Cl^- flux ($J_{\text{Cl(LB)}}$) in the CNT. Values are means \pm SE. Tubular length was 0.34 ± 0.02 mm. Perfusion rates in each period were 7.42 ± 0.65 , 7.07 ± 0.59 , 6.80 ± 0.58 , and 7.02 ± 0.24 nl/min, respectively. * $P < 0.05$, ** $P < 0.01$ as compared with the preceding values.

distal convoluted tubule. This notion is based on the data derived from both osmolar clearance and micropuncture studies. The observations that thiazides inhibit free water clearance without affecting free water reabsorption (1–3) support the view that the drugs inhibit solute transport in the cortical diluting segments. Theoretically, the cortical diluting segments include all the water impermeable nephron segments located distally to the medullary thick ascending limb.

More direct evidence with regard to the site of action of thiazides has been provided by micropuncture studies (4–10). Ullrich et al. (4), using split oil-droplet technique, found that chlorothiazide inhibits sodium transport in both proximal and distal convolutions. Although early free-flow micropuncture studies in the dog (25) and rat (26) have failed to demonstrate the action of thiazides on the proximal tubule, a number of subsequent studies have confirmed that thiazides inhibit sodium chloride reabsorption in this segment (6, 25, 27, 28). However, the inhibition by thiazides of sodium chloride reabsorption in the proximal tubule may not contribute in major way to the increase in urinary excretion of sodium chloride, because the increase in distal delivery of sodium chloride is compensated by the increased reabsorption of sodium chloride in the loop of Henle (6). Free-flow micropuncture (6, 9) and in

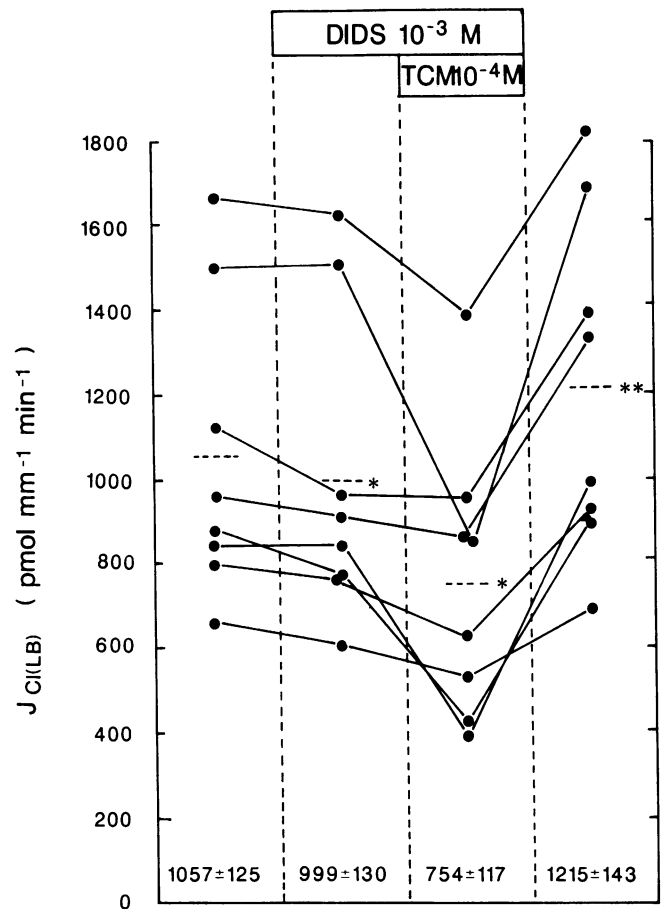


Figure 8. Effects of 10^{-3} M DIDS and 10^{-4} M TCM on $J_{\text{Cl(LB)}}$ in the CNT. Values are means \pm SE. Tubular length was 0.34 ± 0.01 mm. Perfusion rates in each period were 9.09 ± 0.47 , 7.66 ± 0.35 , 6.87 ± 0.19 , and 8.27 ± 0.26 nl/min, respectively. * $P < 0.05$, ** $P < 0.01$ as compared to the preceding values.

vivo microperfusion study (7, 8, 10) in the rat clearly demonstrated that thiazides inhibit sodium chloride transport in the distal tubule.

Although the cortical thick ascending limb has characteristics of the cortical diluting segments, Schlatter et al. (29) reported that thiazide did not affect Cl^- transport in this segment isolated from rabbits. The distal tubule as defined by micropuncture studies is a heterogeneous segment composed of several morphologically as well as functionally distinct segments, including the DCT, the CNT, and the CCD (11–14). In vitro microperfusion of isolated nephron segments is expected to provide a good mean to identify the exact site of action of thiazides in the distal nephron segments. We have chosen the rabbit kidney to examine this issue for the following three reasons. First the isolation of individual distal nephron segments from the kidney without enzymatic treatment is technically feasible only in the rabbit (13–17). Second, information on the functional heterogeneity are available in the distal nephron segments of this species (13–17, 22). Finally, the morphological transition from one segment to another is so abrupt in the rabbit kidney that we may obtain a pure segment without contamination of other segments (11, 12). The results of the present study clearly indicate that TCM inhibits sodium

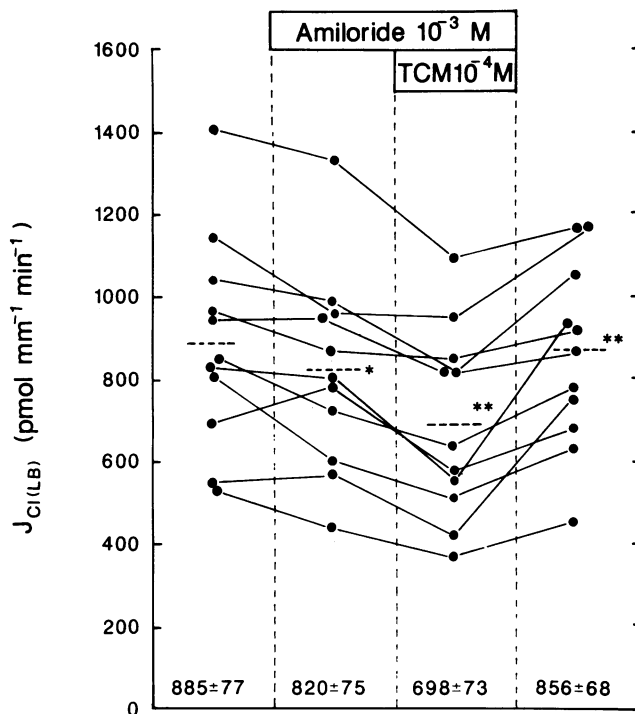


Figure 9. Effects of 10^{-3} M amiloride on $J_{Cl(LB)}$ in the CNT. Values are means \pm SE. Tubular length was 0.42 ± 0.04 mm. Perfusion rates in each periods were 9.24 ± 0.46 , 9.04 ± 0.47 , 8.60 ± 0.43 , and 9.54 ± 0.47 nl/min. * $P < 0.05$, ** $P < 0.01$ as compared to the preceding values.

chloride transport only in the CNT but neither in the DCT nor in the CCD. The absence of the effect of thiazides on the CCD is in good agreement with the observation of Tago et al. (30). Our observations are apparently contradictory to those of Costanzo (9) and Ellison et al. (10), who reported that in the rat chlorothiazide inhibited NaCl transport only in the earliest distal nephron segment but not in late distal segments, which presumably included both the CNT and initial collecting duct. Although we do not know how to explain this discrepancy, species difference in the morphology of the distal nephron segments between rats and rabbits may be, at least in part, responsible for this discrepancy. It should be noted that in the rat distal nephron the transition from one segment to the other is gradual and both intercalated cells and connecting tubule cells are intermingled in early distal portion (12).

Because of the discrepancies between rats and rabbits with regard to the target segment of thiazide action, we have tried to provide more information on the characteristics of the rabbit DCT (Figs. 2 and 3). We confirmed that the DCT has an amiloride sensitive Na^+ transport system in the luminal membrane as does the CNT. The luminal membrane of the DCT may lack a $Na^+-K^+-2Cl^-$ cotransport system that is supposed to be sensitive to furosemide. Even combined administration of furosemide and TCM did not affect Cl^- transport of the DCT. Thus the segment of DCT that we identified is clearly distinct from either the thick ascending limb or the CNT.

Kaissling and Kriz (11) have reported that each distal nephron segment in the rabbit kidney consists of different types of epithelia: the DCT consists of a single population of the distal convoluted tubule cell; the CNT contains the connecting tubule cell and the intercalated cell, and the CCD is

composed of the principal cell and the intercalated cell. On the basis of this morphological distinction of epithelial constituent in each segment, we can determine which type of epithelia is the target of the action of thiazides. The DCT cell is clearly excluded, since the segment of DCT consist of single cell population and TCM was without effect. If the intercalated cell were to be the target, thiazides could have inhibited sodium chloride transport in the CCD as well. Thus, it is most likely that the CNT cell is the target of thiazides. It is of interest to note that in the *Amphiuma* late distal tubule hydrochlorothiazide acts on one of two cell types having conductance to K^+ and Cl^- in the basolateral membrane (31). However, it is unknown at present time whether this type of cell corresponds to the CNT cell in the mammalian kidney.

Mechanism of action of thiazide diuretics. Although it is known that thiazides have a property of carbonic anhydrase inhibitor, the diuretic potency of thiazides is not always in parallel with the potency of carbonic anhydrase inhibition (32). By using free-flow micropuncture in the rat, Kunau et al. (6) compared the effect of chlorothiazide with that of benzolamide, an inhibitor of carbonic anhydrase. They demonstrated that benzolamide did not show any effect on chloride transport in the distal tubule although it inhibited chloride transport in the proximal tubule as did chlorothiazide. Thus, carbonic anhydrase inhibition if not responsible for the mechanism of diuretic action of thiazides. Our observation that TCM inhibits Cl^- transport in the absence of bicarbonate supports this view.

Costanzo and Windhager (7) observed the effect of chlorothiazide on sodium absorption in the superficial distal tubule of the rat kidney perfused in vitro with a sodium chloride-containing and bicarbonate-free solution. They demonstrated that the drug inhibited sodium transport when it was applied in the lumen. This inhibitory effect was associated with a decrease in specific transepithelial resistance without any change in transepithelial voltage. This observation is in accord with the view that thiazides inhibit an electroneutral transport mechanism. Velázquez et al. (33) reported that in the rat distal tubule there is a sodium-dependent chloride transport mechanism. More recently, Wright and his associates (9, 10) demonstrated that thiazides inhibit this process. The observation in the present study that TCM did not inhibit Cl^- flux in the absence of Na^+ supports the view that the drug affects sodium-dependent chloride transport in the CNT.

In the present study, we have analyzed the thiazide inhibitable sodium-dependent chloride transport more in detail in the CNT. Neutral sodium-dependent chloride transport includes at least three different categories; i.e., Na^+-Cl^- cotransport, $Na^+-K^+-2Cl^-$ cotransport, and parallel antiport of Na^+/H^+ and Cl^-/HCO_3^- . We observed in the CNT that furosemide did not affect chloride transport and that TCM inhibited chloride flux in the presence of furosemide. These observations exclude the possible contribution of $Na^+-K^+-2Cl^-$ cotransport system in the action of TCM.

However, it is somewhat difficult to reconcile our data to those obtained in the rat distal tubule (8, 9, 28, 33, 34). Using free flow micropuncture technique in the rat, Duarte et al. (34) found that during intravenous administration of furosemide sodium reabsorption in the distal tubule was not increased, even though the sodium concentration in the distal fluid was increased. It is possible that under these experimental conditions furosemide prevented the increase in sodium reabsorp-

tion that would be expected to occur in response to the increase in luminal sodium concentration. Using in vivo microperfusion of the rat distal tubule, Velázquez et al. (33) demonstrated that furosemide decreased sodium and chloride transport without affecting transmural voltage. One possible explanation for the discrepancy with our observation in regard to furosemide action could be that furosemide acts on the DCT but not on the CNT. But this possibility is unlikely in view of the more recent observation of Velázquez and Wright (9). They reported that chlorothiazide caused a more profound inhibition of sodium chloride transport in the rat distal tubule than did furosemide. When 10^{-3} M chlorothiazide was added to perfusion fluid containing furosemide and amiloride, additional inhibition of both sodium and chloride absorption was noted. On the other hand, addition of 10^{-3} M furosemide to perfusate containing chlorothiazide did not cause additional reduction of sodium or chloride transport. These observations are consistent with the view that both chlorothiazide and furosemide act on the site with furosemide having a lower efficacy. Since Velázquez and Wright (9) demonstrated that bumetanide, a more potent loop diuretic, did not inhibit sodium and chloride absorption in the rat distal tubule, the effect of furosemide on sodium chloride transport in this segment may not be mediated by the action on $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter. Since Stokes (35) also reported that in the urinary bladder of flounder furosemide at 10^{-3} M decreased NaCl fluxes, whereas bumetanide was ineffective, it is highly possible that such peculiar action of furosemide is different among species and is lacking in the rabbit.

Parallel antiport of $\text{Na}^+\text{/H}^+$ and $\text{Cl}^-/\text{HCO}_3^-$ has been shown to exist in various biological membranes (36–41). We assessed whether this type of parallel antiport exists in the CNT and whether TCM affects this system. The results of three different approaches lead us to speculate that there is parallel antiport of $\text{Na}^+\text{/H}^+$ and $\text{Cl}^-/\text{HCO}_3^-$ in the CNT but that this is not a major target of thiazide action. When the $\text{Na}^+\text{/H}^+$ antiport system was inhibited by administration of 10^{-3} M amiloride, the lumen-to-bath Cl^- flux was slightly but significantly decreased. This would suggest that the Cl^- flux is somehow related with the $\text{Na}^+\text{/H}^+$ antiport, although we can not rule out the possibility that a high concentration of amiloride might have caused a nonspecific inhibition of Cl^- transport. The possible existence of $\text{Cl}^-/\text{HCO}_3^-$ or Cl^-/OH^- antiport is supported by the observations that the Cl^- flux was slightly but significantly decreased by either administration of DIDS or elimination of bicarbonate. These observations are consistent with the view that the parallel antiport of $\text{Na}^+\text{/H}^+$ and $\text{Cl}^-/\text{HCO}_3^-$ might be operating in the CNT. However, the component of Cl^- transported via this system may be very small. Since TCM exhibited remarkable inhibitory effects on Cl^- flux under all conditions where 10^{-3} M amiloride or 10^{-4} M DIDS was present in the lumen or bicarbonate was eliminated, the target of TCM action is distinct from the parallel antiport of $\text{Na}^+\text{/H}^+$ and $\text{Cl}^-/\text{HCO}_3^-$. Although the CNT consists of at least two different types of epithelia, we cannot exclude the possibility that both $\text{Na}^+\text{-Cl}^-$ cotransport and parallel antiport system coexist in the one type of cell as observed in the rabbit gall bladder (41). However, in view of the fact that bicarbonate secretion occurs in the CCD (42–45), it is more likely that the parallel antiport of $\text{Na}^+\text{/H}^+$ and $\text{Cl}^-/\text{HCO}_3^-$ exists in the intercalated cell in the CNT. Our observation that TCM inhibited net Cl^- flux only by 31% (Table V) is in accord with the

hypothesis that there are Cl^- transport mechanisms other than $\text{Na}^+\text{-Cl}^-$ cotransport. It is unknown whether $\text{Cl}^-/\text{HCO}_3^-$ exchanger entirely accounts for this component.

Late distal tubule of amphibian kidney is regarded to be comparable to the distal tubule of mammalian kidney (31, 46). Hansen, Schilling and Wiederholt (46) reported that in the late distal tubule of *Amphiuma* 10^{-4} M chlorothiazide added to the lumen caused hyperpolarization of basolateral membrane voltage by -23 mV. More recently, Stanton (31) confirmed this observation in type II cells of the late distal tubule of the *Amphiuma* kidney. Although he suggested the existence of parallel antiport of $\text{Na}^+\text{/H}^+$ and $\text{Cl}^-/\text{HCO}_3^-$, coexistence of simple $\text{Na}^+\text{-Cl}^-$ symport cannot be ruled out.

By excluding other possible mechanisms that explain the Na^+ dependent electroneutral Cl^- transport, we reached the conclusion that a "simple" $\text{Na}^+\text{-Cl}^-$ cotransport system exists in the CNT and that this is the major target of the action of thiazide diuretics. A similar conclusion was made by Stokes et al. (45) based on the studies in the urinary bladder of winter flounder. They demonstrated in this preparation a Na^+ -dependent Cl^- transport system that is not inhibited by high dose of amiloride or DIDS and is little affected by loop diuretics. Although it has been reported in the toad or frog urinary bladder (47, 48) that thiazide inhibits electrogenic Na^+ absorption, this is clearly not the case in the mammalian CNT.

In summary, there are three distinct mechanisms of Na^+ transport in the luminal membrane of the rabbit CNT: (a) amiloride-dependent Na^+ conductance, (b) parallel antiport of $\text{Na}^+\text{/H}^+$ and $\text{Cl}^-/\text{HCO}_3^-$, and (c) simple $\text{Na}^+\text{-Cl}^-$ cotransport. The inhibition of $\text{Na}^+\text{-Cl}^-$ cotransport is the major mechanism involved in the diuretic action of thiazides.

Acknowledgments

We would like to express our thanks to Mrs. Yayoi Ueshima for preparing this manuscript.

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