Effect of S-8666 on Cl^- Transport in the Rabbit Connecting Tubule Perfused in Vitro

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**Department of Pharmacology, National Cardiovascular Center Research Institute, Suita 565, and †Shionogi Research Laboratories, Shionogi & Co., Ltd., Osaka 553*

SHIMIZU, T., NAKAMURA, M. and IMAI, M. *Effect of S-8666 on Cl^- Transport in the Rabbit Connecting Tubule Perfused in Vitro.* Tohoku J. Exp. Med., 1991, **164** (4), 293-298 — S-8666, [6, 7-dichloro-5-(N, N-dimethylsulfamoyl)-2, 3-dihydrobenzofurancarboxylic acid] is a potent diuretic with uricosuric action. Although the major site of action of S-8666 has been proven by the in vitro microperfusion study to be the thick ascending limb of Henle's loop, clearance studies in the rat suggested that this drug has an additional thiazide-like action. To provide direct evidence that S-8666 acts also on distal nephron segments, we examined effect of S-8666 on Cl^- flux across the rabbit connecting tubule perfused in vitro. The drug suppressed the lumen-to-bath Cl^- flux by 96 ± 41 (s.e.) $\text{pmol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1}$ ($n=9$) without affecting transmural voltage. To demonstrate that S-8666 acts on the connecting tubule cell, the target of thiazide diuretics, we compared effects of S-8666 and trichlormethiazide on the basolateral membrane voltage of the connecting tubule cell. Both drugs added to the lumen caused a small but significant hyperpolarization of the basolateral membrane without affecting transmural voltage. We conclude that S-8666 is a unique uricosuric diuretic having actions on both thick ascending limb of Henle's loop and connecting tubule. — uricosuric diuretic; connecting tubule; Cl^- transport; loop diuretic; thiazides

S-8666 [6, 7-dichloro-5-(N, N-dimethylsulfamoyl)-2, 3-dihydrobenzofurancarboxylic acid] is a potent diuretic with uricosuric action (Yonetani et al. 1987; Nakamura et al. 1988). In vitro microperfusion studies from our laboratories have established that the major sites of the diuretic action of S-8666 is the thick ascending limb of Henle's loop (Shimizu et al. 1988a, b). Furthermore, we suggested that this drug also acts on more distal nephron segments (Shimizu et al. 1989a, b). There are two lines of indirect evidence in support of this notion. First, S-8666 caused an increase in urinary Ca^{2+} excretion which was disproportionately smaller than Na^+ excretion (Shimizu et al. 1989a): Second, the com-

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bined administration of S-8666 with furosemide produced an additive natriuretic effect and diminished the hypercalciuric effect of furosemide (Shimizu et al. 1989b). Since anticalciuric effect is the most unique for thiazide diuretics, we speculated that an additional site of action of S-8666 might be common to that of thiazide diuretics.

Shimizu et al. (1988c) reported that in the rabbit trichlormethiazide (TCM) inhibits $\text{Na}^+\text{-Cl}^-$ cotransport in the connecting tubule (CNT). Therefore, we examined effects of S-8666 on Cl^- transport, transmural voltage (V_T), and basolateral membrane voltage (V_B) in the rabbit CNT to provide direct evidence in support of the view that an additional site of action of S-8666 is common to that of thiazide diuretics.

MATERIALS AND METHODS

The rabbit CNTs were perfused in vitro as reported previously (Imai 1979; Shimizu et al. 1988c; Yoshitomi et al. 1988; Muto et al. 1990). The CNT was identified according to the criteria reported previously (Imai 1979). The perfusion solution contained (in mM) 110 mM NaCl, 25 mM NaHCO_3 , 5 mM KCl, 10 mM acetate, 1.0 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 1.8 mM CaCl_2 , 1.0 mM MgCl_2 , 1.5 mM sodium-citrate, 3.5 mM glutamate, 8.3 mM glucose and 5 mM alanine. The perfusate and bath solution were identical in composition. Sodium salts of S-8666 and of TCM, prepared in Shionogi Research Laboratories, were dissolved in the perfusate.

For Cl^- flux measurement, approximately $3 \mu\text{Ci/ml}$ ^{36}Cl was added to the perfusate. All solution were bubbled with 95% O_2 and 5% CO_2 to maintain pH at 7.4. Tubules were perfused at 37°C , with perfusion rate being maintained between 5 and 10 nl/min. Transmural voltage (V_T) was measured as described previously (Shimizu et al. 1988b, c).

Radioactivity of ^{36}Cl was measured with a beta counter (1217 Rack Beta, LKB Wallac, Turku, Finland). The unidirectional Cl^- flux from lumen to bath (J_{Cl}) was calculated as;

$$J_{\text{Cl}} = V_0 [\text{Cl}]_i (1 - [\text{C}^*]_o / [\text{C}^*]_i) / L$$

where $[\text{Cl}]_i$ is the concentration of Cl^- in the perfusate, V_0 is the collection rate, L is the tubular length, and $[\text{C}^*]_i$ and $[\text{C}^*]_o$ are the concentrations of isotope in the perfusate and the collected fluid, respectively.

To obtain direct evidence that both TCM and S-8666 act specifically on the CNT cell, we impaled the CNT cells with conventional microelectrodes filled with 0.5 M KCl while the tubules were perfused in vitro according to the method reported previously (Yoshitomi et al. 1988; Muto et al. 1990). The CNT cells were identified according to the following criteria; (a) the magnitude of basolateral membrane voltage (V_B) is greater than 60 mV, and (b) the positive V_B deflection of greater than 20 mV is generated by abrupt increase in K^+ concentration in the bath from 5 to 50 mM.

Data are reported as means $\pm$ s.e. Values between control and experimental groups were compared by Student's t -test for paired samples. $p < 0.05$ was regarded to be significant.

RESULTS

Nine segments of the CNT were perfused in vitro to examine effects of 1 mM S-8666 added to the lumen on the lumen-to-bath Cl^- flux (J_{Cl}) and transmural voltage (V_T). The results are summarized in Table 1. The perfusion rate remained unchanged throughout the experiments. J_{Cl} was reduced from 948 to

TABLE 1. Effects of S-8666 added to the lumen on lumen-to-bath Cl^- flux (J_{Cl}) and transmural voltage (V_T) in the rabbit connecting tubule^a

	C	E	R	Δ
S-8666 (mM)	0	1	0	
Perfusion rate (nl/min)	8.20 ± 0.58	8.00 ± 0.39	8.68 ± 0.57	
J_{Cl} ($\text{pmol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1}$)	948 ± 83	806 ± 68	855 ± 76	$-96 \pm 41^*$
V_T (mV)	-8.0 ± 2.7	-7.8 ± 2.9	-6.8 ± 2.6	-0.3 ± 0.3

^aThe mean perfused tubular length was 0.44 ± 0.44 mm ($n=9$).

C, control period; E, experimental period; R, recovery period; $\Delta = E - (C+R)/2$. * $p < 0.05$ compared to zero.

$806 \text{ pmol mm}^{-1} \text{ min}^{-1}$ by addition of S-8666 to the lumen. Elimination of the drug increased the value to $855 \text{ pmol} \cdot \text{mm}^{-1} \cdot \text{min}^{-1}$. Since the parameter did not recover to the control level, the data were analyzed by comparing the values in experimental period with means of the values in control and recovery period. The inhibitory effect of S-8666 thus examined was statistically significant ($p < 0.05$). In contrast, the drug did not affect lumen-negative V_T at all.

The segment of CNT contains two different types of epithelia; connecting

Fig. 1. A representative recording of transmural (V_T) and basolateral membrane voltage (V_B) in the rabbit connecting tubule. Trichlormethiazide (TCM) and S-8666 were added to the lumen. Both TCM and S-8666 caused small hyperpolarization of basolateral membrane without appreciable changes in transmural voltage. In this particular experiment, it was confirmed that S-8666 did not show additive effect when it was added with TCM to lumen.

TABLE 2. *Effects of TCM and S-8666 on V_B and V_T of rabbit CNT^a*

	TCM 0.1 mM			S-8666 1 mM		
	C	E	E-C	C	E	E-C
V_B (mV)	-80.3 ± 2.1	-83.0 ± 1.8	$-2.7 \pm 0.6^{**}$	-82.0 ± 2.4	-85.4 ± 1.8	$-3.4 \pm 1.0^{**}$
V_T (mV)	-13.7 ± 2.2	-14.1 ± 2.4	-0.4 ± 0.3	-13.8 ± 2.4	-14.4 ± 2.5	-0.6 ± 0.3

^aData are means of 10 experiments. Drugs were administered to the same tubules successively. TCM, trichlormethiazide; V_B , basolateral membrane voltage; V_T , transmural voltage CNT, connecting tubule; C, control period; E, experimental period. ^{**} $p < 0.01$ as compared to zero.

tubule (CNT) cell and intercalated (IC) cell (Kaissling and Kriz 1979). To examine whether S-8666 acts specifically on the CNT cell, we observed effects of S-8666 on the V_B of this cell type. Fig. 1 shows a representative tracing where effect of S-8666 was compared with that of TCM. In agreement with previous data (Yoshitomi et al. 1988; Muto et al. 1990), we confirmed that V_B of the CNT cell was about -80 mV. Small hyperpolarization occurred when either TCM or S-8666 was added to the lumen. The data of 10 experiments summarized in Table 2 show that both agents caused a small but significant hyperpolarization of basolateral membrane without affecting V_T .

DISCUSSION

In the previous study (Shimizu et al. 1988c), we have established that in the rabbit thiazide diuretics inhibit $\text{Na}^+\text{-Cl}^-$ cotransport by acting exclusively on the CNT. Neither distal convoluted tubule (DCT) nor cortical collecting duct (CCD) is the target of the action of thiazide diuretics. The results of the present study clearly show that in the rabbit CNT 1 mM S-8666 added to the lumen suppresses the lumen-to-bath Cl^- flux without affecting the V_T . Although we did not observe the effect of the drug on Na^+ flux, it is likely that S-8666 inhibits the electroneutral $\text{Na}^+\text{-Cl}^-$ cotransport since it did not affect the V_T .

In rats, it has been reported that thiazide diuretics act on the early distal convoluted tubule (Costanzo 1985; Ellison et al. 1987). However, this does not necessarily deny the hypothesis that the CNT is the target of the action of thiazide diuretics, because in the rat the transition of DCT to CNT is gradual and all types of cells are intermingled even in the early portion of the segment (Crayen and Thoenes 1975).

In this study, we found that both TCM and S-8666 caused small but significant hyperpolarization of basolateral membrane of the CNT cell. Because a moderate Cl^- conductance exists in the basolateral membrane of the CNT cell (Yoshitomi et al. 1988; Muto et al. 1990), the observed hyperpolarization may reflect the reduction of intracellular Cl^- concentration caused by inhibition of Cl^- entry from the apical membrane of this type of cell. S-8666 did not show any

additive effect to TCM. Thus, we conclude that S-8666, like thiazide diuretics, inhibits NaCl reabsorption by acting on the apical membrane of the CNT.

S-8666 at 1 mM suppressed Cl^- flux by 15%, whereas 0.1 mM TCM decreased it by 28% (Shimizu et al. 1988c). It appears that the potency of S-8666 in inhibiting Na^+ - Cl^- cotransport in the CNT is much less than that of TCM, although the available data do not allow us to make exact comparison. NaCl transport across the CNT is mediated by several processes other than the thiazide sensitive Na^+ - Cl^- cotransport. They include amiloride sensitive Na^+ channel, Na^+/H^+ antiport, $\text{HCO}_3^-/\text{Cl}^-$ antiport and simple passive diffusion through paracellular pathway. Especially the $\text{HCO}_3^-/\text{Cl}^-$ antiport which is mainly operated in the β -IC cell may contribute to a large fraction of Cl^- flux in the CNT (Muto et al. 1990). This is the reason why only small fraction of Cl^- flux was inhibited by S-8666 or TCM.

It is fair to say that the decrease in unidirectional Cl^- flux by S-8666 does not necessarily prove the decrease in net Cl^- flux. We cannot exclude the possibility that the drug decreases passive Cl^- permeability through paracellular pathway. In this case, net Cl^- flux might be unchanged even though the lumen-to-bath Cl^- flux is decreased. However, the observation that S-8666 hyperpolarized the basolateral membrane clearly indicate that the drug affects transepithelial Cl^- transport.

Thiazide diuretics are known to have hypocalciuric action, probably resulting from their action on the distal nephron (Costanzo 1985). Shimizu et al. (1991) reported that trichlormethiazide increases Ca^{2+} flux in the presence of PTH. Thus, relative hypocalciuric effect of S-8666 (Shimizu et al. 1989a) may be explained also by its action on the CNT. Since we have also demonstrated that furosemide did not affect Cl^- transport in the CNT (Shimizu et al. 1988c), the additive diuretic effect of S-8666 and furosemide (Shimizu et al. 1989b) may be accounted for by the action of S-8666 on the CNT.

In a previous study (Shimizu et al. 1988b), we have demonstrated definite evidence that S-8666 inhibits NaCl transport in the rabbit thick ascending limb of Henle's loop perfused in vitro. In conjunction with the results of the present study, it is unequivocal that S-8666 has dual sites of action on the thick ascending limb and the CNT. Although we cannot rule out the possibility that S-8666 acts additionally on other distal nephron segments, we have excluded a possible action on the proximal tubule (Shimizu et al. 1988b). The structure-activity relationship responsible for the dual sites of action of S-8666 remains to be established.

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