

## Evidence for conductive Cl<sup>-</sup> pathway in the basolateral membrane of rabbit renal proximal tubule S3 segment.

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### Research Article

The mechanism of Cl<sup>-</sup> exit was examined in the basolateral membrane of rabbit renal proximal tubule S3 segment with double-barreled, ion-selective microelectrodes. After the basolateral Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger was blocked by 2'-disulfonic acid, a bath K<sup>+</sup> step from 5 to 20 mM induced 26.6 mV depolarization and 7.7 mM increase in intracellular Cl<sup>-</sup> activities ([Cl<sup>-</sup>]<sub>i</sub>). K<sup>+</sup> channel blockers, Ba<sup>2+</sup>, and quinine strongly suppressed both the response in cell membrane potentials (V<sub>b</sub>) and in (Cl<sup>-</sup>)<sub>i</sub> to the bath K<sup>+</sup> step, while Cl<sup>-</sup> channel blockers, A9C (1 mM) and IAA-94 (0.3 mM) inhibited only the latter response by 49 and 74%, respectively. By contrast, an inhibitor of K<sup>(+)</sup>-Cl<sup>-</sup> cotransporter, H74, had no effect on the increase in (Cl<sup>-</sup>)<sub>i</sub> to the bath K<sup>+</sup> step. Furosemide and the removal of bath Na<sup>+</sup> were also ineffective, suggesting that (Cl<sup>-</sup>)<sub>i</sub> are sensitive to the cell potential changes. Bath Cl<sup>-</sup> removal in the presence of quinine induced a depolarization of more than 10 mV and a decrease in (Cl<sup>-</sup>)<sub>i</sub>, and IAA-94 inhibited these responses similarly in the bath K<sup>+</sup> step experiments. These results indicate that a significant Cl<sup>-</sup> conductance exists in the basolateral membrane of this segment and functions as a Cl<sup>-</sup> exit mechanism.

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# Evidence for Conductive $\text{Cl}^-$ Pathway in the Basolateral Membrane of Rabbit Renal Proximal Tubule S3 Segment

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## Abstract

The mechanism of  $\text{Cl}^-$  exit was examined in the basolateral membrane of rabbit renal proximal tubule S3 segment with double-barreled, ion-selective microelectrodes. After the basolateral  $\text{Cl}^-/\text{HCO}_3^-$  exchanger was blocked by 2'-disulfonic acid, a bath  $\text{K}^+$  step from 5 to 20 mM induced 26.6 mV depolarization and 7.7 mM increase in intracellular  $\text{Cl}^-$  activities ( $[\text{Cl}^-]_i$ ).  $\text{K}^+$  channel blockers,  $\text{Ba}^{2+}$ , and quinine strongly suppressed both the response in cell membrane potentials ( $V_b$ ) and in  $(\text{Cl}^-)_i$  to the bath  $\text{K}^+$  step, while  $\text{Cl}^-$  channel blockers, A9C (1 mM) and IAA-94 (0.3 mM) inhibited only the latter response by 49 and 74%, respectively. By contrast, an inhibitor of  $\text{K}^+-\text{Cl}^-$  cotransporter, H74, had no effect on the increase in  $(\text{Cl}^-)_i$  to the bath  $\text{K}^+$  step. Furosemide and the removal of bath  $\text{Na}^+$  were also ineffective, suggesting that  $(\text{Cl}^-)_i$  are sensitive to the cell potential changes. Bath  $\text{Cl}^-$  removal in the presence of quinine induced a depolarization of more than 10 mV and a decrease in  $(\text{Cl}^-)_i$ , and IAA-94 inhibited these responses similarly in the bath  $\text{K}^+$  step experiments. These results indicate that a significant  $\text{Cl}^-$  conductance exists in the basolateral membrane of this segment and functions as a  $\text{Cl}^-$  exit mechanism. (*J. Clin. Invest.* 1993. 92:1229–1235.) Key words: renal proximal tubule • S3 segment • microelectrodes •  $\text{Cl}^-$  conductance •  $\text{K}^+-\text{Cl}^-$  cotransporter

## Introduction

Reabsorption of NaCl along the renal proximal tubules is considered to be mediated by both paracellular and transcellular routes (1, 2). The mechanism of transcellular  $\text{Na}^+$  transport has been well characterized, including apical  $\text{Na}^+/\text{H}^+$  exchanger and basolateral  $\text{Na}^+$ -dependent transporters (3). By contrast, the cellular mechanism of  $\text{Cl}^-$  transport is poorly understood. Since a  $\text{Cl}^-$  conductance in the basolateral membrane of the proximal tubules is reported to be negligibly small (4, 5), an electroneutral process has been proposed as a  $\text{Cl}^-$  exit mechanism (6–8). In this regard some investigators have concluded that  $\text{K}^+-\text{Cl}^-$  cotransporter exists in the basolateral membrane of rabbit proximal tubule S2 segment (9, 10). Their conclusion was mainly based on the observations that intracel-

lular  $\text{Cl}^-$  activities ( $[\text{Cl}^-]_i$ )<sup>1</sup> did not change in response to the current application from luminal perfusion pipette, but did change upon the stepwise alterations in bath  $\text{K}^+$  concentrations. However in the absence of precise knowledge about the mechanism of apical  $\text{Cl}^-$  transport, it would be difficult to interpret the data obtained by the current injection from lumen. Moreover the overall cell responses in the S2 segment could be also complicated by the coexistence of several transporters such as electrogenic  $\text{Na}^+-\text{HCO}_3^-$  cotransporter or  $\text{Na}^+$ -dependent and  $\text{Na}^+$ -independent  $\text{Cl}^-/\text{HCO}_3^-$  exchanger (3, 11–13).

Recent electrophysiological studies have revealed axial heterogeneity in the mechanism for bicarbonate reabsorption along the proximal tubules; in particular the rabbit proximal tubule S3 segment has been shown not to contain the  $\text{Na}^+-\text{HCO}_3^-$  cotransporter (14). Instead the  $\text{Na}^+$ -independent  $\text{Cl}^-/\text{HCO}_3^-$  exchanger functions as only one major  $\text{HCO}_3^-$  transporter in the basolateral membrane of this segment (15, 16). The transport system of this segment thus clarified would be more preferable when a missing mechanism of  $\text{Cl}^-$  exit were to be examined. This prompted us to reinvestigate the mechanism of basolateral  $\text{Cl}^-$  transport in the rabbit proximal tubule S3 segment, care being taken to avoid the influence of luminal process by analyzing the nonperfused, lumenally collapsed tubules.

## Methods

The experiments were performed on isolated tubules from female New Zealand white rabbits (1.5–2.5 kg body wt) as previously described (13, 16). In brief, thin sections of kidney were obtained after killing the animals and were stored in ice-cold Ringer's solution. S3 segments of both superficial and juxtamedullary nephrons were identified from their transition to the thin descending limb of Henle's loop. Tubule fragments within 1 mm length from this transition were dissected and transferred to the perfusion chamber mounted on an inverted microscope (IMT-2; Olympus, Tokyo, Japan). To avoid the influence from the luminal process, we analyzed only the nonperfused, lumenally collapsed tubules, as described in a previous study (17), and they were perfused only peritubularly at a rate of  $\sim 10$  ml/min with prewarmed (38°C) experimental solutions. The compositions of these solutions are listed in Table I, and fluid exchanges were completed within 1 s by using a rotary valve as reported previously (14, 16).

Cell membrane potentials ( $V_b$ ) and  $(\text{Cl}^-)_i$  or cell pH ( $\text{pH}_i$ ) were measured with double-barreled ion-selective microelectrodes using a dual-channel electrometer (Duo 773; WPI, Sarasota, FL). To minimize the generation of liquid-junction potentials, an open-tip reference electrode filled with saturated KCl solution contacted the chamber solution to form a flowing-boundary junction. In this reference system

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1. Abbreviations used in this paper: A9C, anthracene-9-carboxylate;  $(\text{Cl}^-)_i$ , intracellular  $\text{Cl}^-$  activities; DIDS, 4,4'-diisothiocyanostilbene-2, 2'-disulfonic acid; DPC, diphenylamine-2-carboxylate; IAA-94, indanyl-oxyacetic acid 94; NPPB, 5-nitro-2-(3-phenylpropyl-amino)-benzoate;  $\text{pH}_i$ , cell pH;  $V_b$ , cell membrane potentials.

Table I. Composition of Perfusion Solutions

	A <sup>‡</sup>	B <sup>‡</sup>	C	D	E
	Control	high-K <sup>+</sup>	Cl <sup>-</sup> -free	Na <sup>+</sup> -free	Na <sup>+</sup> -free high-K <sup>+</sup>
Na <sup>+</sup>	144	129	144	—	—
NMDG <sup>+*</sup>	—	—	—	144	129
K <sup>+</sup>	5	20	5	5	20
Ca <sup>2+</sup>	1.5	1.5	5	1.5	1.5
Mg <sup>2+</sup>	1	1	1	1	1
Cl <sup>-</sup>	125	125	—	125	125
Gluconate <sup>-</sup>	—	—	132	—	—
HCO <sub>3</sub> <sup>-</sup>	25	25	25	25	25
H <sub>2</sub> PO <sub>4</sub> <sup>-</sup>	2	2	2	2	2
SO <sub>4</sub> <sup>2-</sup>	1	1	1	1	1
D-glucose	5.5	5.5	5.5	5.5	5.5

All concentrations in mmol/liter. \* *N*-methyl-D-glucamine. ‡ When Ba<sup>2+</sup> was added to these solutions, H<sub>2</sub>PO<sub>4</sub><sup>-</sup> and SO<sub>4</sub><sup>2-</sup> were replaced by Cl<sup>-</sup>. The pH of all the solutions was adjusted to 7.4 by bubbling with 5% CO<sub>2</sub>/95% O<sub>2</sub> gas.

the changes in the liquid-junction potentials upon bath fluid changes were virtually negligible, and no correction was made for the measured cell potentials. Ion-selective, double-barreled microelectrodes were constructed as previously described (14, 18), using the ionophore cocktail 24902 for the Cl<sup>-</sup> electrodes and 95297 for the pH electrodes, respectively (Fluka, Neu-Ulm, Germany). Average slopes and resistances of the electrodes were 54.0±0.5 mV and 1.8±0.2 × 10<sup>11</sup> Ω for the Cl<sup>-</sup> electrodes (*n* = 37), and 58.9±0.7 mV and 4.8±0.7 × 10<sup>10</sup> Ω for the pH electrodes (*n* = 5). The calculation of (Cl<sup>-</sup>)<sub>i</sub> was based on the same principles as in the previous studies (15, 18). The ionophore cocktail for the Cl<sup>-</sup> electrodes used in the present studies was recently shown to have better selectivity for Cl<sup>-</sup> than the conventional ligand (18). Indeed upon the bath Cl<sup>-</sup> removal, (Cl<sup>-</sup>)<sub>i</sub> decreased to a very low level (less than 3 mmol/liter), indicating that the problems of anion interference would be minimal, if any, in our experimental conditions. The electrodes were inserted into Ag/AgCl pellet holders and mounted on a micromanipulator (MW-2; Narishige, Tokyo, Japan) for manually puncturing the tubules.

H-74, furosemide, and 5-nitro-2-(3-phenylpropyl-amino)-benzoate (NPPB) were kind gifts from Hoechst (Frankfurt aM, Germany). Diphenylamine-2-carboxylate (DPC) was obtained from Fluka, anthracene-9-carboxylate (A9C) was from Sigma Immunochemicals (St. Louis, MO), and indanyl-oxyacetic acid 94 (IAA-94) from Res. Biochem., Inc. (Natick, MA). When the effects of these chemicals were tested, tubules were incubated with them for at least 1 min. NPPB and DPC were dissolved in DMSO (final concentration of 0.01%), and IAA-94 was dissolved in ethanol (final concentration of 0.02%). When they were used, equivalent concentrations of either DMSO or ethanol were added to the control perfusates. 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) was from Sigma Immunochemicals. All the other chemicals were of reagent grade and purchased from Wako Junyaku (Osaka, Japan). Throughout the paper, mean values±SEM are given. The paired *t* test was applied, and *P* < 0.05 was accepted as statistically significant.

## Results

*Effect of bath K<sup>+</sup> step on V<sub>b</sub> and (Cl<sup>-</sup>)<sub>i</sub> in absence and presence of DIDS.* Since (Cl<sup>-</sup>)<sub>i</sub> were reported to be sensitive to the alterations of bath K<sup>+</sup> concentrations in the S2 segment (9, 10), we first examined the effect of bath K<sup>+</sup> step (from 5 to 20 mM,

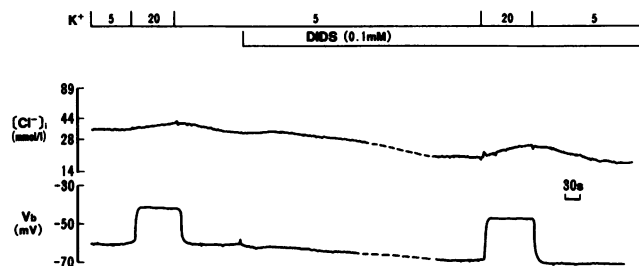


Figure 1. Effect of bath K<sup>+</sup> step on V<sub>b</sub> and (Cl<sup>-</sup>)<sub>i</sub> in the absence and presence of DIDS. The top line gives the changes in bath K<sup>+</sup> concentrations in mmol/liter (solutions A and B of Table I). The tubule was incubated with DIDS for about 15 min.

solutions A and B) on V<sub>b</sub> and (Cl<sup>-</sup>)<sub>i</sub> in the lumenally collapsed tubules from rabbit proximal S3 segment. As shown in Fig. 1, this K<sup>+</sup> step induced a sudden depolarization and a gradual increase in (Cl<sup>-</sup>)<sub>i</sub>. To exclude a latent influence of the basolateral Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger, we repeated the bath K<sup>+</sup> step before and after the addition of DIDS. As can be seen in Fig. 1 and summarized in Table II, 0.1 mM DIDS gradually hyperpolarized V<sub>b</sub> and decreased (Cl<sup>-</sup>)<sub>i</sub>. About 10–15 min after the addition of DIDS the cell parameters reached a new steady state, where V<sub>b</sub> hyperpolarized by ~ 10 mV and (Cl<sup>-</sup>)<sub>i</sub> decreased by ~ 13 mM. The cell responses to bath K<sup>+</sup> step were rather enhanced after DIDS: +19.1±1.1 vs. +26.6±1.3 mV in ΔV<sub>b</sub> (*n* = 11, *P* < 0.01), and +5.9±0.7 vs. +7.7±0.8 mM in Δ(Cl<sup>-</sup>)<sub>i</sub> (*n* = 11, *P* < 0.05), respectively. Fig. 2 shows that the activity of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger was almost completely inhibited by DIDS as previously reported (19). Approximately 15 min after the addition of DIDS, the pH<sub>i</sub> response to bath Cl<sup>-</sup> removal was reduced from +0.38±0.02 to +0.03±0.01 U (*n* = 5, *P* < 0.01). Of note is that DIDS virtually did not change the steady-state pH<sub>i</sub> (7.17 vs. 7.18; *n* = 5, *P* < 0.10). These results suggest that the cell responses to bath K<sup>+</sup> step are independent of the Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger. The reasons for the hyperpolarization or the decrease in (Cl<sup>-</sup>)<sub>i</sub> after DIDS were not apparent by this time (see Discussion), but the inhibition of this exchanger might be advantageous in that one could investigate the mechanism of Cl<sup>-</sup> exit without the influence of other transporters. Therefore all the subsequent experiments were performed in the tubules incubated with DIDS for at least 10 min after the successful cell impalement.

Table II. Cell Responses to Bath K<sup>+</sup> Step in the Absence and Presence of DIDS (0.1 mM)

	V <sub>b</sub> <sup>*</sup>	(Cl <sup>-</sup> ) <sub>i</sub> <sup>*</sup>	ΔV <sub>b</sub> <sup>‡</sup>	Δ(Cl <sup>-</sup> ) <sub>i</sub> <sup>‡</sup>
	mV	mmol/liter	mV	mmol/liter
Control	-56.1±1.6	33.3±1.7	+19.1±1.1	+5.9±0.7
DIDS	-66.5±2.0	20.0±1.4	+26.6±1.3	+7.7±0.8
<i>P</i> value	<0.01	<0.01	<0.01	<0.05

Data are mean values ± SE from 11 tubules. \* Steady-state membrane potentials and intracellular Cl<sup>-</sup> activities. ‡ Shift of steady-state membrane potentials. † Shift of intracellular Cl<sup>-</sup> activities, measured at 90 s after bath fluid change. The significant levels refer to control and DIDS values of the same column.

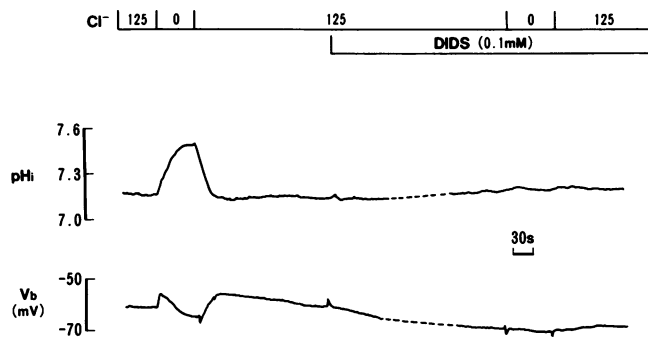


Figure 2. Effect of bath  $\text{Cl}^-$  removal on  $V_b$  and  $\text{pH}_i$  in the absence and presence of DIDS. Details as in Fig. 1, but solutions A and C were used.

**Mechanism of the cell responses to bath  $\text{K}^+$  step.** Theoretically the increase in  $(\text{Cl}^-)_i$  to bath  $\text{K}^+$  step could be mediated by the following mechanisms: (a) parallel  $\text{K}^+$  and  $\text{Cl}^-$  conductances, (b)  $\text{K}^+$ - $\text{Cl}^-$  cotransport, and (c)  $\text{Na}^+/\text{K}^+/2\text{Cl}^-$  cotransport. The first possibility was tested by separately applying  $\text{K}^+$  and  $\text{Cl}^-$  channel blockers. The effects of two structurally unrelated  $\text{K}^+$  channel blockers,  $\text{Ba}^{2+}$  and quinine, are shown in Fig. 3 and summarized in Table III. The addition of 3 mM  $\text{Ba}^{2+}$ , after producing a large depolarization of  $\sim 40$  mV and a gradual increase in  $(\text{Cl}^-)_i$ , completely blocked the  $V_b$  and  $(\text{Cl}^-)_i$  responses to bath  $\text{K}^+$  step, while 0.4 mM quinine inhibited both responses by more than 80%. The effects of  $\text{Cl}^-$  chan-

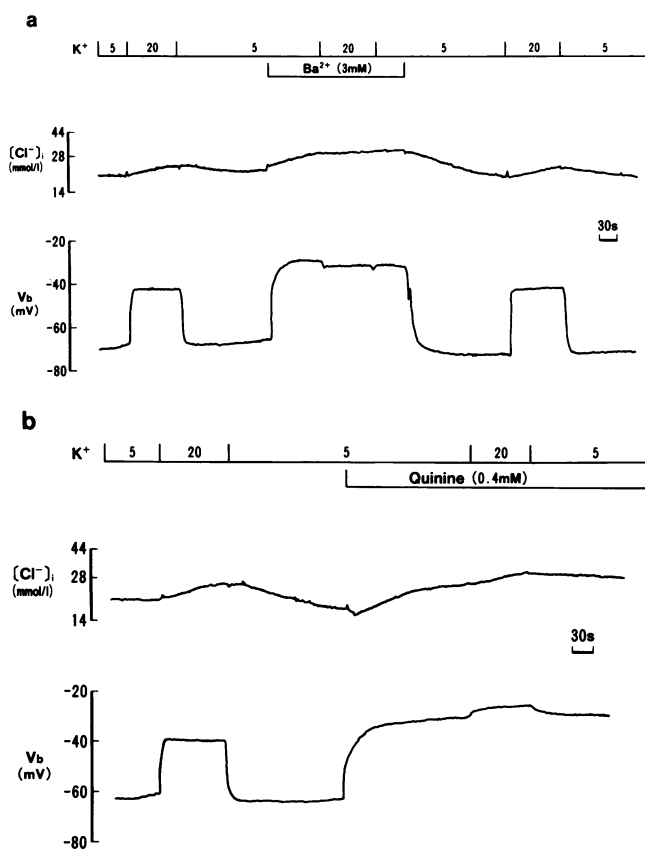


Figure 3. *a* and *b*. Cell responses to bath  $\text{K}^+$  step in the presence of (a) 3 mM  $\text{BaCl}_2$  or (b) 0.4 mM quinine-HCl. Details as in Fig. 1.

Table III. Effects of  $\text{Ba}^{2+}$  (3 mM) and Quinine (0.4 mM) on the Cell Responses to Bath  $\text{K}^+$  Step

	$V_b$	$(\text{Cl}^-)_i$	$\Delta V_b$	$\Delta(\text{Cl}^-)_i$
	mV	mmol/liter	mV	mmol/liter
Control	$-66.0 \pm 2.5$	$20.4 \pm 1.6$	$+26.9 \pm 1.8$	$+6.9 \pm 0.9$
$\text{Ba}^{2+}$	$-26.5 \pm 2.4$	$34.9 \pm 3.5$	$-1.9 \pm 0.4$	$-1.0 \pm 0.4$
<i>P</i> value	<0.01	<0.01	<0.01	<0.01
Control	$-66.7 \pm 1.5$	$17.9 \pm 1.3$	$+26.1 \pm 1.4$	$+6.6 \pm 0.3$
Quinine	$-33.5 \pm 2.5$	$24.8 \pm 2.0$	$+4.7 \pm 0.9$	$+1.1 \pm 0.3$
<i>P</i> value	<0.01	<0.05	<0.01	<0.01

Details as in Table II, but data are from six tubules for both the experiments with  $\text{Ba}^{2+}$  and quinine.

nel blockers are shown in Fig. 4 and Table IV. A9C (1 mM) and IAA-94 (0.3 mM) did not affect the  $V_b$  response, but did inhibit the  $(\text{Cl}^-)_i$  response to bath  $\text{K}^+$  step by  $48.9 \pm 4.1$  and  $74.2 \pm 2.9\%$ , respectively. The concentration dependence of the IAA-94 effect on this  $(\text{Cl}^-)_i$  response was determined as shown in Fig. 5, indicating that much higher concentrations were required in this segment compared with the previous reports in other tissues (20, 21). Of the other  $\text{Cl}^-$  channel blockers that could be examined, neither NPPB (10  $\mu\text{M}$ ) nor DPC (0.1 mM) had any effect on the cell responses to bath  $\text{K}^+$  step (data not shown). These observations are in agreement with the existence of parallel  $\text{K}^+$  and  $\text{Cl}^-$  conductances.

To test the second possibility, H74, a recently discovered specific inhibitor of  $\text{K}^+$ - $\text{Cl}^-$  cotransporter, was used. As shown in Fig. 6, 0.1 mM H74, which has been shown to inhibit the  $\text{K}^+$ - $\text{Cl}^-$  cotransport in human red blood cells by more than 50% (22), had no effect on the cell responses to bath  $\text{K}^+$  step. The average responses from three tubules were:  $+27.9 \pm 0.3$  vs.

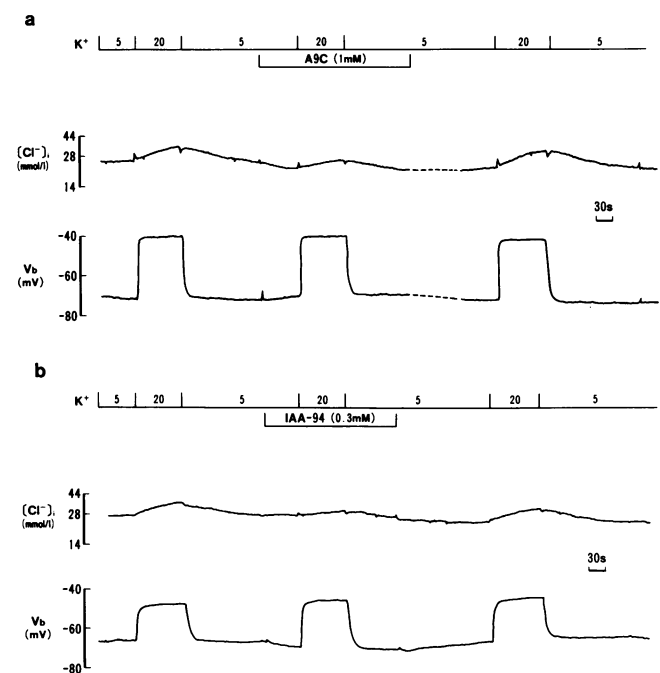


Figure 4. *a* and *b*. Cell responses to bath  $\text{K}^+$  step in the presence of (a) 1 mM A9C or (b) 0.3 mM IAA-94. Details as in Fig. 1.

Table IV. Effects of A9C (1 mM) and IAA-94 (0.3 mM) on the Cell Responses to K<sup>+</sup> Step

	V <sub>b</sub>	[Cl <sup>-</sup> ] <sub>i</sub>	ΔV <sub>b</sub>	Δ[Cl <sup>-</sup> ] <sub>i</sub>
	mV	mmol/liter	mV	mmol/liter
Control	-66.7±1.7	26.0±0.8	+26.3±2.0	+10.7±1.0
A9C	-66.2±1.8	25.8±1.8	+25.8±2.0	+5.5±0.6
<i>P</i> value				<0.01
Control	-59.4±2.3	17.5±1.4	+20.6±0.6	+7.1±0.6
IAA-94	-60.8±2.6	18.3±1.2	+22.0±0.7	+1.9±0.3
<i>P</i> value				<0.01

Details as in Table II, but data are from five tubules in the experiments with A9C and from four tubules in those with IAA-94.

+27.3±0.6 mV in ΔV<sub>b</sub> (NS), and +9.1±0.2 vs. +9.1±0.2 mM in Δ[Cl<sup>-</sup>]<sub>i</sub> (NS). These results do not support the operation of K<sup>+</sup>-Cl<sup>-</sup> cotransporter.

The third possibility was tested either by the application of loop diuretics or by the total substitution of bath Na<sup>+</sup> with NMDG<sup>+</sup>. The addition of furosemide (50 μM) did not affect both the V<sub>b</sub> and [Cl<sup>-</sup>]<sub>i</sub> responses to bath K<sup>+</sup> step as shown in Fig. 7. The average Δ[Cl<sup>-</sup>]<sub>i</sub> response was +7.7±1.2 mM in the absence and +7.6±2.1 mM in the presence of furosemide (*n* = 3, NS). Similarly the removal of bath Na<sup>+</sup> did not affect the [Cl<sup>-</sup>]<sub>i</sub> response, though the V<sub>b</sub> response to bath K<sup>+</sup> step was slightly reduced in the absence of Na<sup>+</sup> as shown in Fig. 8. The average responses from four tubules were: +23.3±2.5 vs. +16.4±1.3 mV in ΔV<sub>b</sub> (*P* < 0.01), and +6.8±0.4 vs. +5.5±0.4 mM in Δ[Cl<sup>-</sup>]<sub>i</sub> (NS). The reason for the smaller V<sub>b</sub> response in the Na<sup>+</sup>-free condition is not clear, but these results indicate that the operation of Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>-</sup> cotransporter is unlikely.

**Electrogenicity of the cell responses to bath Cl<sup>-</sup> removal.** The preceding results strongly suggest the operation of parallel K<sup>+</sup> and Cl<sup>-</sup> conductances as the mechanism of cell responses to bath K<sup>+</sup> step. The existence of Cl<sup>-</sup> conductance should, in turn, induce a significant depolarization in response to bath Cl<sup>-</sup> removal. However if the size of Cl<sup>-</sup> conductance is relatively small, it would be difficult to detect the depolarization because the large basolateral K<sup>+</sup> conductance would mask the small Cl<sup>-</sup> current. The latter prediction was the case as shown in Fig. 9, where the bath Cl<sup>-</sup> removal induced the significant depolarization only in the presence of quinine. In this series 0.8 mM quinine was used, which inhibited the V<sub>b</sub> response to bath K<sup>+</sup> step by more than 90% (data not shown). Since the prolonged blockade of K<sup>+</sup> conductance could increase the cell

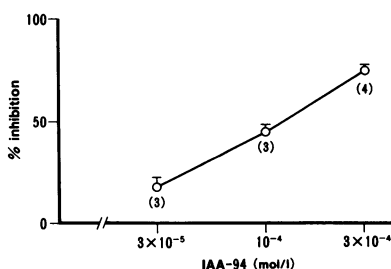


Figure 5. Concentration dependence of the effect of IAA-94 on the [Cl<sup>-</sup>]<sub>i</sub> response to bath K<sup>+</sup> step. Numbers of observations are given in parentheses.

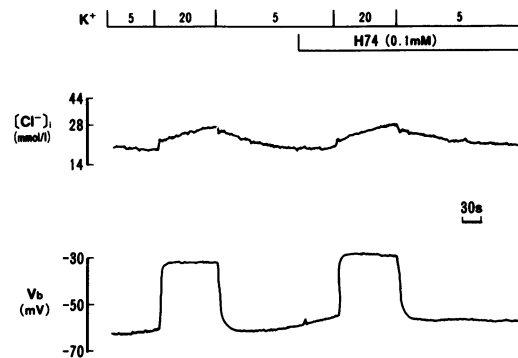


Figure 6. Cell responses to bath K<sup>+</sup> step in the presence of 0.1 mM H74. Details as in Fig. 1.

volume, which might next activate the volume-sensitive channels (23, 24), 10 mM mannitol was added simultaneously with quinine to suppress the potential cell swelling. The average V<sub>b</sub> response to bath Cl<sup>-</sup> removal from five tubules was -1.0±0.7 mV in the absence and +15.2±2.9 mV in the presence of quinine (*P* < 0.01). The initial rates of [Cl<sup>-</sup>]<sub>i</sub> decrease to bath Cl<sup>-</sup> removal (d[Cl<sup>-</sup>]<sub>i</sub>/dt) determined from the first 10-s response were not affected by quinine (-0.24±0.05 vs. -0.25±0.04 mM/s, NS). These results confirm that a small Cl<sup>-</sup> conductance really exists in the basolateral membrane of S3 segment. Finally, the effect of IAA-94 (0.3 mM) on these responses to Cl<sup>-</sup> removal was examined as shown in Fig. 10 and Table V, and, as expected, both the V<sub>b</sub> and [Cl<sup>-</sup>]<sub>i</sub> responses were inhibited by this blocker. The close inspection of the V<sub>b</sub> traces in Fig. 10 reveals that the depolarizing response to bath Cl<sup>-</sup> removal is composed of the initial fast component and the subsequent slower component. The reason for this type of V<sub>b</sub> response is not apparent, but the inhibitory effect of IAA-94 is limited in the slower V<sub>b</sub> response, suggesting that only this component reflects the true Cl<sup>-</sup> current. The initial small depolarization (usually less than 5 mV) might be due to the circular current generated by bath Cl<sup>-</sup> substitution. This current would be minimal in the lumen-collapsed tubules, but its influence on V<sub>b</sub> could be enhanced after quinine increased the resistance of the basolateral membrane. The addition of IAA-94 decreased the initial rates of V<sub>b</sub> changes (dV<sub>b</sub>/dt) of the slower component by 58.8%, and the initial rates of [Cl<sup>-</sup>]<sub>i</sub> decrease (d[Cl<sup>-</sup>]<sub>i</sub>/dt) by 60.3% (*n* = 6, Table V). These values were comparable to

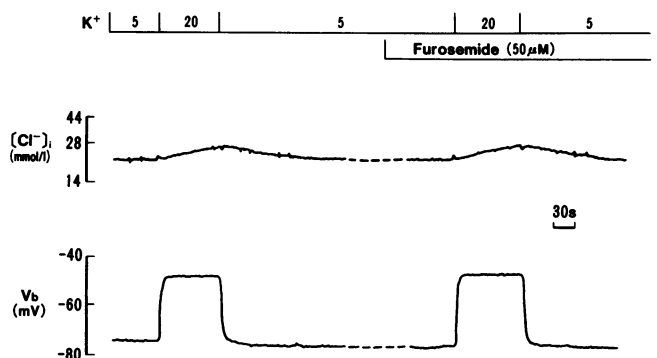


Figure 7. Cell responses to bath K<sup>+</sup> step in the presence of 50 μM furosemide. Details as in Fig. 1.

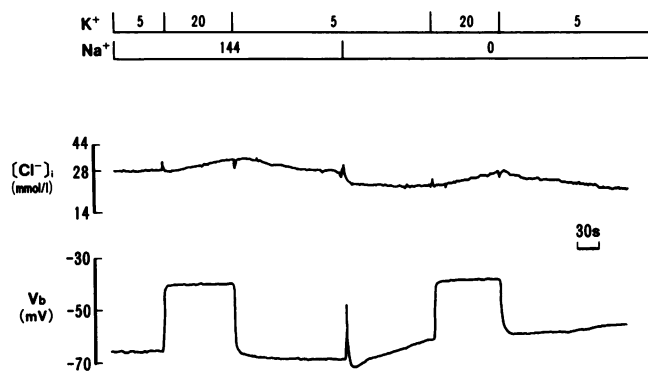


Figure 8. Cell responses to bath  $K^+$  step in the presence and absence of bath  $Na^+$ . Details as in Fig. 1, but solutions D and E were also used.

the effect of IAA-94 in the bath  $K^+$  step experiments (74.2% inhibition, see Table IV).

## Discussion

**Evidence for  $Cl^-$  conductance.** The following observations clearly indicate that the  $Cl^-$  conductance exists in the basolateral membrane of rabbit proximal tubule S3 segment: (a) the sudden increase in bath  $K^+$  concentrations induced the depolarization and the increase in  $(Cl^-)_i$ , both of which were inhibited by the  $K^+$  channel blockers,  $Ba^{2+}$  and quinine; (b) the  $Cl^-$  channel blockers, A9C and IAA-94, inhibited the  $(Cl^-)_i$  response without affecting the  $V_b$  response to bath  $K^+$  step; and (c) bath  $Cl^-$  removal in the presence of quinine induced the significant depolarization and the decrease in  $(Cl^-)_i$ , and IAA-94 also inhibited these responses.

The effects of the  $K^+$  channel blockers on the cell responses to bath  $K^+$  step indicate that  $(Cl^-)_i$  are sensitive to the changes in  $V_b$ . Besides its well-known effect as the  $K^+$  channel blocker,  $Ba^{2+}$  has been suggested to have other effects such as an inhibition of  $K^+-Cl^-$  cotransporter (25). However since quinine, another structurally unrelated  $K^+$  channel blocker (24, 26), had similar effects as  $Ba^{2+}$ , we conclude that the  $(Cl^-)_i$  response to bath  $K^+$  step can be attributed to the cell potential changes.

The inhibition of the  $(Cl^-)_i$  response to bath  $K^+$  step by  $Cl^-$  channel blockers supports the above interpretation that  $Cl^-$

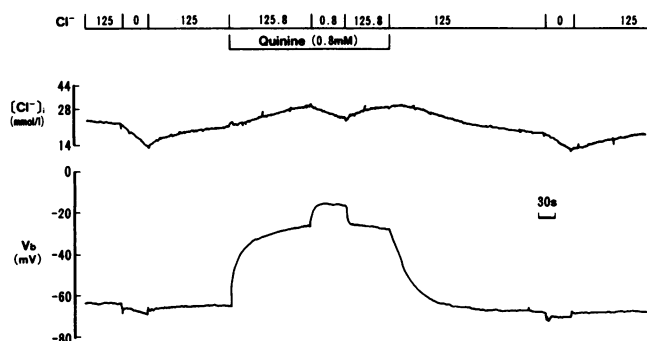


Figure 9. Cell responses to bath  $Cl^-$  removal in the absence and presence of 0.8 mM quinine-HCl. Mannitol (10 mM) was also added simultaneously with quinine. Note a significant depolarization only in the presence of quinine.

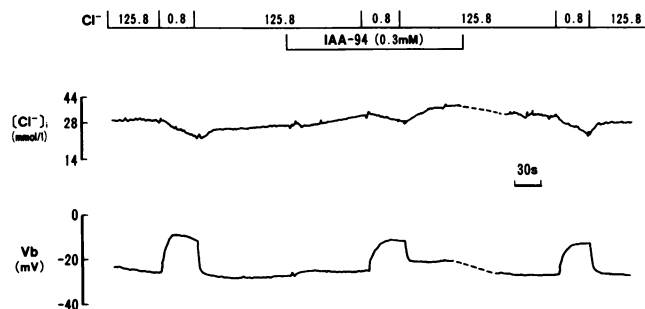


Figure 10. Effect of 0.3 mM IAA-94 on the cell responses to bath  $Cl^-$  removal in the presence of 0.8 mM quinine-HCl and 10 mM mannitol. Note IAA-94 inhibited both the slower phase of the depolarization and the decrease in  $(Cl^-)_i$ .

moves through the conductive pathway across the basolateral membrane. In this segment only IAA-94 at the high concentrations and A9C were effective, whereas DIDS, DPC, or NPPB were ineffective at the concentrations used in the present studies. This kind of relative insensitivity to the blockers is, however, recently reported on the small  $Cl^-$  channels in HT-29 cells (27, 28), and may simply imply the diversity of  $Cl^-$  channels in the epithelial cells (27–30).

Regarding the mechanisms other than the  $Cl^-$  conductance, the observation that H74, the inhibitor of  $K^+-Cl^-$  cotransporter (22), had no effect on the cell responses to bath  $K^+$  step speaks against the possibility of  $K^+-Cl^-$  cotransporter. Since we do not know much about the property of this cotransporter, it would be rather difficult to completely reject the existence of this transporter. However if the  $K^+-Cl^-$  cotransporter is electroneutral as previously reported (31), the electrogenic response to bath  $Cl^-$  removal detected in the presence of quinine also cannot be attributed to the operation of this cotransport. Another possibility of  $Na^+/K^+/2Cl^-$  cotransporter seems to be even less likely, since furosemide had no effect on the cell responses to bath  $K^+$  step, and in addition, the removal of bath  $Na^+$  did not affect the  $(Cl^-)_i$  response to bath  $K^+$  step.

With the  $Cl^-$  conductance, the electrogenic response was clearly shown when the bath  $Cl^-$  was removed in the presence of quinine. The failure to detect the significant depolarization in the absence of quinine suggests that this conductance could be relatively small, since the large  $K^+$  conductance would tend to shunt the other small currents. The  $Cl^-$  channel blocker, IAA-94, inhibited not only the depolarization but also the rate

Table V. Effect of IAA-94 (0.3 mM) on the Cell Responses to Bath  $Cl^-$  Removal in the Presence of Quinine (0.8 mM)

	$V_b$	$(Cl^-)_i$	$dV_b/dt^*$	$d(Cl^-)_i/dt^*$
	mV	mmol/liter	mV/s	mmol/liter $\times$ s
Control	$-19.9 \pm 1.2$	$31.1 \pm 1.4$	$+2.5 \pm 0.2$	$-0.38 \pm 0.04$
IAA-94	$-18.2 \pm 2.0$	$33.6 \pm 1.6$	$+1.1 \pm 0.1$	$-0.16 \pm 0.04$
P value			<0.01	<0.01

Data are from six tubules. \* Initial rates of potential changes measured in the first 5 s of the slower depolarizing phase. † Initial rates of decrease in  $(Cl^-)_i$  measured within the first 10 s after bath fluid change.

of  $(\text{Cl}^-)_i$  decrease to bath  $\text{Cl}^-$  removal, strongly supporting the existence of  $\text{Cl}^-$  conductance. Because the rates of  $(\text{Cl}^-)_i$  decrease to bath  $\text{Cl}^-$  removal were not affected by quinine, the argument that another  $\text{Cl}^-$  conductance might have been newly activated by the blockade of  $\text{K}^+$  channel can be largely discarded. Moreover the simultaneous addition of mannitol with quinine would suppress the possible cell swelling, and indeed we did not detect any changes in the cell volume estimated from the tubule diameters during the administration of quinine. These results indicate that the same  $\text{Cl}^-$  conductance mediates both the electrogenic response to  $\text{Cl}^-$  removal and the  $(\text{Cl}^-)_i$  response to  $\text{K}^+$  step. The comparative inhibitory effects of IAA-94 on these two responses also support this conclusion.

*Comparison with previous studies.* Although some reports indicate the presence of cell swelling-activated  $\text{Cl}^-$  channels (32, 33), most of the previous electrophysiological studies suggest that there is no significant  $\text{Cl}^-$  conductance in the basolateral membrane of the proximal tubules at physiological conditions (4, 5), and therefore electroneutral process has been suggested as the  $\text{Cl}^-$  exit mechanism (6–8). Our present studies are limited in the S3 segment and may not be directly compared with the previous studies mostly done in the S2 segment. However it could be possible that the presence of large  $\text{K}^+$  conductance as well as the influence of other transporters might have masked the existence of small  $\text{Cl}^-$  conductance even in the S2 segment. In fact, the DIDS-insensitive  $\text{Cl}^-$  conductance has been reported in the basolateral membrane vesicles from the rat proximal tubules (34), and recently the presence of  $\text{Cl}^-$  channel in the basolateral membrane of *Ambystoma* proximal tubules is also reported in an abstract form (35).

Some investigators have proposed the operation of  $\text{K}^+$ - $\text{Cl}^-$  cotransporter in the basolateral membrane of rabbit proximal S2 segment (9, 10). However, the technique of current injection from the luminal side seems to be problematic, as already mentioned in the Introduction. This critique will be justified, especially if both the apical and the basolateral membranes are conductive to  $\text{Cl}^-$ . Indeed the existence of  $\text{Cl}^-$  channel in the apical membrane of proximal tubules has been recently suggested (36, 37). These considerations as well as our present results have cast the doubt on the operation of  $\text{K}^+$ - $\text{Cl}^-$  cotransporter, though it remains to be determined whether a similar  $\text{Cl}^-$  conductance as we found in the S3 segment exists in the other segments of proximal tubules.

*Physiological role of the  $\text{Cl}^-$  conductance.* Our control measurements before the addition of DIDS clearly indicate that the intracellular  $\text{Cl}^-$  concentrations in the proximal tubules are far above the electrochemical equilibrium, confirming the results from previous studies (6, 7, 9, 10, 17). According to this electrochemical gradient, the  $\text{Cl}^-$  ion should be driven out of the cell through the conductive pathway. The decrease in  $(\text{Cl}^-)_i$  after the DIDS addition supports this view, since the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger normally transports  $\text{Cl}^-$  into the cell (15). After the inhibition of this exchanger, the cell parameters will come closer to the equilibrium for  $\text{Cl}^-$ . This prediction coincides with the hyperpolarization observed after DIDS. On the other hand, the basolateral  $\text{K}^+$  channel in the proximal tubules is reported to be voltage sensitive (38). This may explain the enhanced  $V_b$  response to bath  $\text{K}^+$  step in the presence of DIDS.

The next important question, how much this basolateral  $\text{Cl}^-$  conductance contributes to the overall transcellular  $\text{NaCl}$  reabsorption, cannot be answered by the present studies. How-

ever, this conductance may play a major role in the transcellular  $\text{Cl}^-$  transport provided that a significant  $\text{Cl}^-$  entry step exists in the apical membrane. To determine the exact physiological role of this conductance the entire mechanism of apical  $\text{Cl}^-$  transport should be clarified in the future studies.

Another important issue is about the regulatory mechanism of this  $\text{Cl}^-$  conductance. Since we have recently demonstrated that the basolateral  $\text{Cl}^-/\text{HCO}_3^-$  exchanger in this segment is stimulated by cAMP (17), it might be interesting to examine the role of cAMP. In fact the recent patch-clamp study suggests the existence of cAMP-activated  $\text{Cl}^-$  channel in the basolateral membrane of the *Ambystoma* proximal tubules (35).

In summary we have identified the significant  $\text{Cl}^-$  conductance in the basolateral membrane of rabbit proximal tubule S3 segment. This conductance most likely functions as the  $\text{Cl}^-$  exit mechanism at physiological conditions.

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## References

1. Rector, R. C., Jr. 1983. Sodium, bicarbonate, and chloride absorption by the proximal tubule. *Am. J. Physiol.* 244(Renal Fluid Electrolyte Physiol. 13):F461–F471.
2. Alpern, R. J., K. J. Howlin, and P. A. Preisig. 1985. Active and passive components of chloride transport in the rat proximal convoluted tubule. *J. Clin. Invest.* 76:1360–1366.
3. Alpern, R. J. 1990. Cell mechanisms of proximal tubule acidification. *Physiol. Rev.* 70:79–114.
4. Bello-Reuss, E. 1982. Electrical properties of the basolateral membrane of the straight portion of the rabbit proximal renal tubule. *J. Physiol. (Lond.)* 326:49–63.
5. Burckhardt, B. C., K. Sato, and E. Frömter. 1984. Electrophysiological analysis of bicarbonate permeation across the peritubular cell membrane of rat kidney proximal tubule. I. Basic observations. *Pfluegers Arch. Eur. J. Physiol.* 401:34–42.
6. Shindo, T., and K. R. Spring. 1981. Chloride movement across the basolateral membrane of proximal tubule cells. *J. Membr. Biol.* 58:35–42.
7. Guggino, W. B., E. L. Boulpaep, and G. Giebisch. 1982. Electrical properties of chloride transport across the Necturus proximal tubule. *J. Membr. Biol.* 65:185–196.
8. Baum, M., and C. A. Berry. 1984. Evidence for neutral transcellular  $\text{NaCl}$  transport and neutral basolateral chloride exit in the rabbit proximal convoluted tubule. *J. Clin. Invest.* 74:205–211.
9. Sasaki, S., K. Ishibashi, N. Yoshiyama, and T. Shiigai. 1988.  $\text{KCl}$  co-transport across the basolateral membrane of rabbit renal proximal straight tubules. *J. Clin. Invest.* 81:194–199.
10. Ishibashi, K., F. C. Floyd, Jr., and C. A. Berry. 1990. Chloride transport across the basolateral membrane of rabbit proximal convoluted tubules. *Am. J. Physiol.* 258(Renal Fluid Electrolyte Physiol. 27):F1569–F1578.
11. Yoshitomi, K., B. C. Burckhardt, and E. Frömter. 1985. Rheogenic sodium-bicarbonate cotransport in the peritubular cell membrane of rat renal proximal tubule. *Pfluegers Arch. Eur. J. Physiol.* 405:360–366.
12. Sasaki, S., and N. Yoshiyama. 1988. Interaction of chloride and bicarbonate transport across the basolateral membrane of rabbit proximal straight tubule. *J. Clin. Invest.* 81:1004–1011.
13. Seki, G., and E. Frömter. 1992. Acetazolamide inhibition of basolateral base exit in rabbit renal proximal tubule S2 segment. *Pfluegers Arch. Eur. J. Physiol.* 422:60–65.
14. Kondo, Y., and E. Frömter. 1987. Axial heterogeneity of sodium-bicarbonate cotransport in proximal straight tubule of rabbit kidney. *Pfluegers Arch. Eur. J. Physiol.* 410:481–486.
15. Kondo, Y., and E. Frömter. 1990. Evidence for chloride/bicarbonate exchange mediating bicarbonate efflux from S3 segments of rabbit renal proximal tubule. *Pfluegers Arch. Eur. J. Physiol.* 415:726–733.
16. Seki, G., and E. Frömter. 1990. The chloride/base exchanger in the basolateral cell membrane of rabbit renal proximal tubule S3 segment requires bicarbonate to operate. *Pfluegers Arch. Eur. J. Physiol.* 417:37–41.

17. Seki, G., S. Taniguchi, S. Uwatoko, K. Suzuki, and K. Kurokawa. 1993. Effect of parathyroid hormone on acid-base transport in rabbit renal proximal tubule S3 segment. *Pfluegers Arch. Eur. J. Physiol.* 423:7-13.
18. Kondo, Y., and E. Frömter. 1989. A new double-barrelled, ionophore-based microelectrode for chloride ions. *Pfluegers Arch. Eur. J. Physiol.* 414:663-668.
19. Nakhoul, N. L., L. K. Chen, and W. F. Boron. 1990. Intracellular pH regulation in rabbit S3 proximal tubule: basolateral Cl-HCO<sub>3</sub> exchange and Na-HCO<sub>3</sub> cotransport. *Am. J. Physiol.* 258(*Renal Fluid Electrolyte Physiol.* 27):F371-F381.
20. Landry, D. W., M. H. Akabas, C. Redhead, A. Edelman, E. J. Cragoe, Jr., and Q. Al-Awqati. 1989. Purification and reconstitution of chloride channels from kidney and trachea. *Science (Wash. DC)*. 244:1469-1472.
21. Iijima, K., L. Lin, A. Nasjletti, and M. S. Goligorsky. 1991. Intracellular ramification of endothelin signal. *Am. J. Physiol.* 260(*Cell Physiol.* 29):C982-C992.
22. Ellory, J. C., A. C. Hall, S. O. Ody, H. C. Englert, D. Mania, and H. J. Lang. 1990. Selective inhibitors of KCl cotransport in human red cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 262:215-218.
23. Filipovic, D., and H. Sackin. 1992. Stretch- and volume-activated channels in isolated proximal tubule cells. *Am. J. Physiol.* 262(*Renal Fluid Electrolyte Physiol.* 31):F857-F870.
24. Hazama, A., and Y. Okada. 1988. Ca<sup>2+</sup> sensitivity of volume-regulatory K<sup>+</sup> and Cl<sup>-</sup> channels in cultured human epithelial cells. *J. Physiol. (Lond.)*. 402:687-702.
25. Greger, R., and E. Schlatter. 1983. Properties of the basolateral membrane of the cortical thick ascending limb of Henle's loop of rabbit kidney. *Pfluegers Arch. Eur. J. Physiol.* 396:325-334.
26. Bokvist, K., P. Rorsman, and P. A. Smith. 1990. Block of ATP-regulated and Ca<sup>2+</sup>-activated K<sup>+</sup> channels in mouse pancreatic  $\beta$ -cells by external tetraethylammonium and quinine. *J. Physiol. (Lond.)*. 423:327-342.
27. Kubitz, R., R. Warth, N. Allert, K. Kunzelmann, and R. Greger. 1992. Small-conductance chloride channels induced by cAMP, Ca<sup>2+</sup>, and hypotonicity in HT<sub>29</sub> cells: ion selectivity, additivity, and stilbene sensitivity. *Pfluegers Arch. Eur. J. Physiol.* 421:447-454.
28. Fischer, H., K. M. Kreusel, B. Illek, T. E. Machen, U. Hegel, and W. Claus. 1992. The outwardly rectifying Cl<sup>-</sup> channel is not involved in cAMP-mediated Cl<sup>-</sup> secretion in HT-29 cells: evidence for a very-low-conductance Cl<sup>-</sup> channel. *Pfluegers Arch. Eur. J. Physiol.* 422:159-167.
29. Anderson, M. P., and M. J. Welsh. 1991. Calcium and cAMP activate different chloride channels in the apical membrane of normal and cystic fibrosis epithelia. *Proc. Natl. Acad. Sci. USA*. 88:6003-6007.
30. Cliff, W. H., and R. A. Frizzell. 1990. Separate Cl<sup>-</sup> conductances activated by cAMP and Ca<sup>2+</sup> in Cl<sup>-</sup>-secreting epithelial cells. *Proc. Natl. Acad. Sci. USA*. 87:4956-4960.
31. Lauf, P. K. 1985. K<sup>+</sup>:Cl<sup>-</sup> cotransport: sulfhydryls, divalent cations, and the mechanism of volume activation in a red cell. *J. Membr. Biol.* 88:1-13.
32. Welling, P. A., and R. G. O'Neil. 1990. Cell swelling activates basolateral membrane Cl and K conductances in rabbit proximal tubule. *Am. J. Physiol.* 258(*Renal Fluid Electrolyte Physiol.* 27):F951-F962.
33. Schild, L., P. S. Aronson, and G. Giebisch. 1991. Basolateral transport pathways for K<sup>+</sup> and Cl<sup>-</sup> in rabbit proximal tubule: effects on cell volume. *Am. J. Physiol.* 260(*Renal Fluid Electrolyte Physiol.* 29):F101-F109.
34. Grassl, S. M., P. D. Holohan, and C. R. Ross. 1987. Cl<sup>-</sup>-HCO<sub>3</sub><sup>-</sup> exchange in rat renal basolateral membrane vesicles. *Biochim. Biophys. Acta*. 905:475-484.
35. Segal, A. S., and E. L. Boulpaep. 1992. cAMP-activated chloride channel on the basolateral membrane of renal proximal tubule. *J. Am. Soc. Nephrol.* 3:819. (Abstr.)
36. Lipkowitz, M. S., and R. G. Abramson. 1989. Modulation of the ionic permeability of renal cortical brush-border membranes by cAMP. *Am. J. Physiol.* 257(*Renal Fluid Electrolyte Physiol.* 26):F769-F776.
37. Suzuki, M., T. Morita, K. Hanaoka, Y. Kawaguchi, and O. Sakai. 1991. A Cl<sup>-</sup> channel activated by parathyroid hormone in rabbit renal proximal tubule cells. *J. Clin. Invest.* 88:735-742.
38. Kawahara, K., M. Hunter, and G. Giebisch. 1987. Potassium channels in *Neoturus* proximal tubule. *Am. J. Physiol.* 253(*Renal Fluid Electrolyte Physiol.* 22):F488-F494.