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Regulation of Intracellular Chloride Activity During Perfusion with Hypertonic Solutions in the *Necturus* Proximal Tubule

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Summary. In a previous study we presented evidence that chloride transport across the basolateral membrane in *Necturus* proximal tubule cells occurs predominantly via exchange for both Na^+ and HCO_3^-. In this study the regulation of intracellular chloride was further examined in the doubly-perfused kidney preparation using conventional and chloride-sensitive microelectrodes. Application of hypertonic basolateral solutions containing 80 mM raffinose stimulated an efflux of chloride such that chloride activity remained unchanged at control levels. Membrane potential did not change in these experiments. Inhibition of Cl^- exit across the basolateral cell membrane by removal of either HCO_3^- or Na^+ from the perfusion solution resulted in a significant increase in intracellular chloride activity, a^i_{Cl}, when basolateral osmolarity was raised. Hypertonic basolateral solutions also produced a significant rise in a^i_{Cl} in the presence of SITS.

This study provides further evidence that chloride is transported across the basolateral cell membrane in exchange for both Na^+ and HCO_3^-. Since this exchange mechanism is activated in response to hypertonic solutions, these studies suggest a functional role for this exchanger in the regulation of a^i_{Cl} in the *Necturus* proximal tubule cell during volume changes.

Key Words chloride-bicarbonate exchange · basolateral membrane · *Necturus* proximal tubule · hypertonic conditions

Introduction

A large portion of filtered chloride is reabsorbed by the proximal tubule of the kidney. Kimura and Spring [10] have provided evidence that a significant part of proximal tubule chloride reabsorption occurs via a transcellular pathway. Chloride enters this pathway by way of an apical cell membrane Na^+/Cl^- cotransport system that raises the intracellular chloride above electrochemical equilibrium [13]. Despite the fact that there is an electrochemical gradient favoring chloride movement across the basolateral membrane, it has been shown recently [8] that most chloride transport across the basolateral membrane occurs by way of a carrier that translocates chloride out of the cell in exchange for bicarbonate and sodium. Whether this exchange system serves solely to effect transcellular chloride transport or whether it also has another function is not known.

Volume regulation has been studied intensively in recent years, and in many systems studied chloride plays an important role in the regulatory mechanism [11]. Whether a cell merely shrinks, or regulates its volume by uptake of chloride with a cation as described in several systems, one would expect intracellular chloride activity to rise under hypertonic conditions. To examine the role of $Na^+ - HCO_3^-/Cl^-$ exchange in the regulation of intracellular chloride, we have measured intracellular chloride activity during perfusion of hypertonic solutions with and without maneuvers that have been shown to block this exchanger. Part of this material has been presented in abstract form [7].

Materials and Methods

Kidney Preparation

Adult male and female *Necturus* were obtained from Connecticut Valley Biological Supply Co. (Southampton, MA), kept in an aquarium at 12 °C for at least one month prior to use and fed live goldfish. The kidneys were doubly-perfused via the aortic and portal circulations as described previously [6]. The kidney surface was continuously superfused. The composition of the perfusion solutions is listed in Table 1. The peritubular perfusion was changed using a four-way valve placed near the site of caudal vein cannulation, such that solution changes occurred within 12 sec. The simultaneously-made superfusion solution changes were identical to those of the caudal vein. Solutions were made hypertonic by adding 80 mM raffinose. In addition, 0.1 mg of 0.1% Hercules shade #2 (N. Kohnstamn and Co., New York, NY) was added initially to the basolateral control solution in order to confirm by its disappearence that solution changes had occurred in the perfused tubule. Studies were performed after the dye was washed out. Only early proximal tubules [9] were studied.

Table 1. Concentrations of solutes[a] in *Necturus* perfusion solutions (mM)

Ion	1 Control	2 Control + 80 mM raffinose	3 HCO_3^- free (pH 7.6)	4 HCO_3^- free (pH 7.6) + 80 mM raffinose	5 Low Na^+	6 Low Na^+ + 80 mM raffinose
Na^+	100.5	100.5	96.1	96.1	0.5	0.5
K^+	2.5	2.5	2.5	2.5	2.5	2.5
Ca^{++}	1.8	1.8	1.8	1.8	1.8	1.8
Mg^{++}	1.0	1.0	1.0	1.0	1.0	1.0
Cl^-	98.1	98.1	98.1	98.1	98.1	98.1
HCO_3^-	10.0	10.0	–	–	10.0	10.0
$H_2PO_4^-$	0.5	0.5	0.5	0.5	0.5	0.5
BDA^+[b]	–	–	–	–	90.0	90.0
$HEPES^-$	–	–	5.6	5.6	–	–
HEPES	–	–	4.4	4.4	–	–
Raffinose	–	80.0	–	80.0	–	80.0
TMA^+[c]	–	–	–	–	10.0	10.0

[a] Additionally all solutions contained 400 mg/liter glucose, 2,000 units/liter Heparin and 15 g/liter polyvinylpyrrolidone.
[b] BDA^+–BIS(2-hydroxyethyl)dimethylammonium chloride.
[c] TMA^+-Tetramethylammonium hydroxide

Basolateral Cell Membrane Potential Differences

Conventional single-barreled microelectrodes made on a horizontal microelectrode puller (Model PD-5, Narishige Scientific Instruments, Tokyo, Japan) from 1.2 mm OD and 0.5 mm ID fiber containing glass capillaries (Frederick Haer and Co., Brunswick, ME) and filled with 1 M KCl were used. The resistance of the electrodes ranged from 50 to 80×10^6 Ω and the tip potentials were less than 5 mV. The basolateral cell membrane potential difference (V_{bl}) was recorded by one of the two channels of a very high input impedance electrometer (Model F223, WPI Instruments, New Haven, CT) and was measured with reference to a 3-M KCl Agar bridge placed in the superfusion solution on the surface of the kidney. Connection of the microelectrode and the reference electrode to the electrometer was made by Ag/AgCl half cells. Criteria for acceptable intracellular impalements were: (i) an abrupt change in potential from baseline, (ii) an intracellular voltage that remained constant within 2 mV and (iii) a return of the voltage to the original baseline when the microelectrode was withdrawn from the cell.

Intracellular Chloride Activity

Chloride ion specific electrodes were made from the same glass capillaries as the conventional microelectrodes using a modification of the technique of Fujimoto and Kubota [5]. Electrodes were dipped for 3 sec in a 0.1% solution of a silicone polymer (1107 Fluid, Dow Corning Corp, Midland, MI) in acetone then heated at 300 °C for 30 min and then back-filled with a small amount of Cl^--specific ion exchanger (Corning #477315). The "thick slurry technique" [12] was used to bevel the electrodes, and only chloride electrodes with tip diameters less than 1 μM were back-filled with 0.5 M KCl and immersed in 100 mM NaCl for several hours before use. The chloride-sensitive electrodes had an average slope of 54 mV/decade ($n=38$) in pure KCl solutions ranging from 10 to 100 mM KCl. Their resistance was $5–8 \times 10^{10}$ Ω, and the response time was on the order of 1 sec. The selectivity of the Cl^- electrode to HCO_3^- was 10:1, and corrections for intracellular interference by non-chloride ions were not applied.

Intracellular Cl^- activity, a^i_{Cl}, was measured continuously in the proximal tubule cells by simultaneous continuous impalements with a conventional KCl and a chloride-specific microelectrode. Both electrodes were maintained within the cells of the same tubule during perfusion of the control solution and during changes in the apical or basolateral solutions. Voltages from the two electrodes were subtracted by a dual, differential, very high impedance electrometer (Model F-223 Dual Channel Electrometer). Intracellular chloride activity was calculated from the following equation:

$$a^i_{Cl} = a^{bl}_{Cl}\, 10^{\left[\frac{V_{Cl} - V_{bl}}{S}\right]}$$

where a^i_{Cl} and a^{bl}_{Cl} are intracellular and basolateral Cl^- activities, respectively, and V_{Cl} the voltage change in the Cl^- electrode from the basolateral solution to the inside of the cell. V_{bl} is the voltage change in the conventional KCl microelectrode.

All results are expressed as the mean ± SEM. Significance levels were evaluated by paired t test.

Results

During control perfusions the mean intracellular chloride activity for all the tubules ($n=25$) was 12.4 ± 1.0 mM and the mean basolateral membrane potential -60 ± 3 mV. These values are very similar to those obtained previously [8].

Effect of Hypertonic Solutions on Intracellular Chloride Activity

Raising the osmolarity of the basolateral solution from 210 mOsm (solution 1) to 290 mOsm (solution 2) resulted in no significant change in intracellular chloride activity or basolateral membrane potential (Table 2). Exposure to hypertonic solutions (solution 2) of apical *and* basolateral membranes simultaneously also resulted in no significant change in intracellular chloride activity or membrane potential (Table 2), so all further experiments were

Table 2. Effect of hypertonic solutions on intracellular chloride activity

	a^i_{Cl} (mM)	V_{bl} (mV)	$V_{bl}-E_{Cl}$ (mV)[a]
Control	11.7±1.5	−65±3	−16.5±3.8
Basolateral control and 80 mM raffinose	12.1±1.3	−65±3	−17.5±3.8
P (n=12)	NS	NS	NS
Control	16.6±2.6	−48±6	− 9.2±2.8
Basolateral and apical control and 80 mM raffinose	13.7±1.0	−55±6	−12.0±4.0
P (n=5)	NS	NS	NS

[a] The sign convention was chosen so that a negative sign for $V_{bl}-E_{Cl}$ indicates that the electrochemical driving force is directed outwards across the basolateral membrane.

Table 3. Effect of hypertonic basolateral solutions on intracellular chloride activity in the absence of bicarbonate or in the presence of SITS

	a^i_{Cl} (mM)	V_{bl} (mV)	$V_{bl}-E_{Cl}$ (mV)
HCO_3^- free pH 7.6[a]	12.2±1.1	−56±3	− 9.6±3.2
HCO_3^- free pH 7.6 and basolateral 80 mM raffinose	21.2±1.6	−52±4	−20.1±4.4
P (n=8)	<0.001	NS	< 0.005
SITS[b]	9.9±1.6	−62±5	− 9.6±3.4
SITS and basolateral 80 mM raffinose	17.1±2.0	−58±6	−19.7±3.6
P (n=5)	<0.001	NS	NS

[a] HEPES was used to replace HCO_3^- in both apical and basolateral solutions and the pH was kept constant at control levels.
[b] SITS was applied at a concentration of 5×10^{-4} M in the basolateral solutions starting 15 min prior to the experiment.

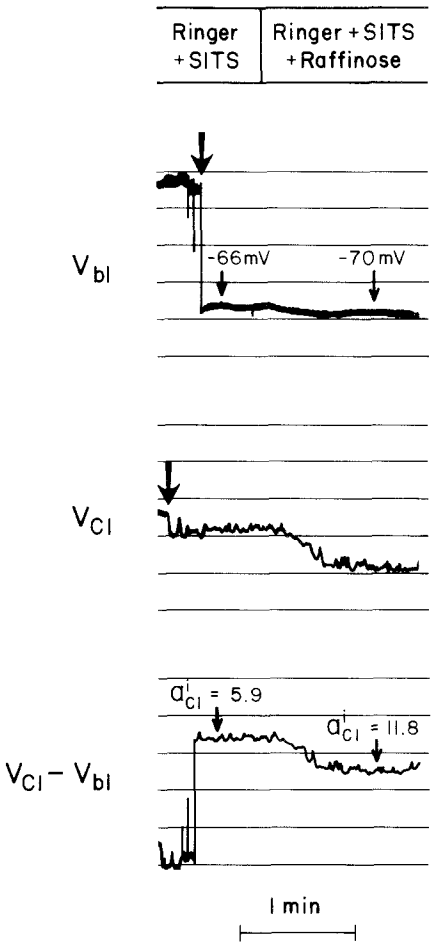

Fig. 1. Typical recording of intracellular chloride activity during shrinkage in the presence of SITS basolaterally. V_{bl} is the basolateral membrane potential and V_{Cl}, the signal from the chloride-sensitive electrode. $V_{Cl}-V_{bl}$, the difference between those potentials, reflects the value of a^i_{Cl}. As shown, a^i_{Cl} increased from 5.9 to 11.8 in response to hypertonic conditions in this SITS-treated tubule. Large arrows mark time of cell impalements

made with basolateral changes only. To see whether a large basolateral chloride efflux was involved in maintaining intracellular chloride activity constant in the face of an 80-mM osmotic gradient, the next series of experiments was designed to test whether the efflux of chloride occurred across the basolateral cell membrane via a Na^+- and HCO_3^--dependent exchange mechanism.

Effect of Hypertonic Solutions on Intracellular Chloride Activity in Bicarbonate-Free Solutions or in the Presence of SITS

To determine if HCO_3^- in the perfusion solution is required for intracellular Cl^- regulation, HCO_3^- was removed from both apical and basolateral perfusion solutions at constant pH (solution 3).

When 80 mM raffinose was added to the basolateral HCO_3^--free solution at constant pH (solution 4), intracellular chloride activity rose from 12.2±1.1 mM to 21.2±1.6 mM despite a significant increase in the electrochemical driving force ($V_{bl}-E_{Cl}$) in the direction of chloride efflux (Table 3). Membrane potential showed no significant change. This is consistent with our previously reported observation that HCO_3^- in the basolateral solution is required for the efflux of chloride.

Further evidence for the interaction of HCO_3^- with the transport of Cl^- was obtained in experiments using SITS, a known inhibitor of anion exchange in red blood cells [1]. SITS was applied to the basolateral solution only at a concentration of 5×10^{-4} M for 15 min prior to the experiment. Because the chloride electrode signal was sensitive

Table 4. Effect of hypertonic basolateral solutions on intracellular chloride activity in the absence of basolateral sodium

	a^i_{Cl} (mM)	V_{bl} (mV)	$V_{bl}-E_{Cl}$ (mV)
Control	10.9 ± 1.3	-61 ± 4	-11.2 ± 3.9
	$P<0.05$	$P<0.05$	$P<0.01$
Basolateral low Na^+	13.9 ± 1.9	-50 ± 5	-6.5 ± 3.5
	$P<0.05$	NS	NS
Basolateral low Na^+ and basolateral 80 mM raffinose	19.3 ± 2.8	-45 ± 6	-8.7 ± 3.9
P ($n=8$)			

to SITS in the concentration used, the V_{Cl} used to calculate a^i_{Cl} (*see* Materials and Methods) was the difference between the voltage recorded by the chloride electrode in a control Ringer *in vitro* and that recorded inside the cell. Addition of 80 mM raffinose to the basolateral solution in the presence of SITS resulted in a significant rise in a^i_{Cl} from 9.9 ± 1.6 mM to 17.1 ± 2.0 mM (Table 3). The basolateral membrane potential was not changed significantly by this maneuver. A representative recording is shown in Fig. 1.

Effect of Hypertonic Solutions on Intracellular Chloride Activity in the Absence of Basolateral Na^+

We have previously shown that Na^+ is required in the basolateral solution for the efflux of chloride across the basolateral membrane [8]. Therefore the next set of experiments was designed to see if regulation of intracellular chloride in the presence of hypertonic solutions required the presence of Na^+ in the basolateral solution. A reduction in the Na^+ activity in the basolateral solution from 76 to 0.5 mM (solution 5) was made and then a solution in which Na^+ activity was similarly reduced but to which 80 mM raffinose had been added (solution 6) was perfused. A continuous record of intracellular chloride activity was made during each of these solution changes for all of the tubules reported. A reduction in the Na^+ in the basolateral solution caused a significant increase in a^i_{Cl} from 10.9 ± 1.3 mM to 13.9 ± 1.9 mM, which then rose significantly to 19.3 ± 2.8 mM when 80 mM raffinose was added to the low Na^+ basolateral solution (Table 4). The membrane potential fell significantly from -61 ± 4 mV to -50 ± 5 mV when the low Na^+ solution was perfused but did not change significantly when 80 mM raffinose was present in the basolateral solution. These experiments provide further evidence that the presence of Na^+ in the basolateral solution influences intracellular Cl^- activity and that chloride regulation in the presence of hypertonic basolateral solutions is occurring via a Cl^- efflux across the basolateral membrane that requires Na^+ in the basolateral solution.

Discussion

Previous studies suggest that chloride exit across the basolateral membrane of *Necturus* proximal tubule occurs predominantly via exchange for sodium and bicarbonate [8]. To further delineate the possible role of this exchange pathway we measured intracellular chloride activity under control conditions and during perfusion with hypertonic solutions. This protocol was chosen because a rise in intracellular chloride activity might be expected whether the cells merely shrink or whether they volume regulate (*see* below). Our studies have shown that when hypertonic solutions are perfused on the basolateral side (or on both sides) intracellular chloride activity remains remarkably constant. If maneuvers previously shown to block $Na^+-HCO_3^-/Cl^-$ exchange are used [8], intracellular chloride activity rises significantly when hypertonic basolateral solutions are applied. Thus, this exchanger appears to regulate intracellular chloride activity when bathing solutions are made hypertonic.

Several volume regulatory mechanisms have been described in detail in the last few years. A feature common to most of these systems is that chloride enters cells that have been shrunk by hypertonic solutions and leaves those that have been swelled. This is accomplished, for example, by coupled Na^+-K^+-Cl^- entry or K^+-Cl^- loss in some systems [11] or parallel cation/H^+ and HCO_3^-/Cl^- exchangers in others [2, 4].

In particular, in *Necturus* gallbladder, Fisher, Persson and Spring [4] have demonstrated brisk cell volume regulation in response to shrinkage through activation of parallel Na^+/H^+ and Cl^-/HCO_3^- exchange pathways in the apical membrane. In their study cell volume initially decreased to 85.6% of the control volume within 40 sec and then regulated back to 96.2% of that volume over the next 90 sec despite the continued presence of hypertonic bathing media. During the volume regulatory phase in these studies [4], intracellular chloride activity hardly changed. This is surprising, because if volume regulation occurred solely by way of NaCl entry as is suggested, chloride activity should have risen substantially unless a simulta-

neous chloride efflux through a separate pathway had also been stimulated. In fact, one can calculate that if all volume regulation could be attributed to NaCl entry, the intracellular chloride activity would have risen by about 6–7 mM, or more than 25% (assuming an intracellular activity coefficient of 0.76), whereas the measured rise was less than 0.5%. Thus, a large chloride efflux was most likely occurring.

In our studies we do not know what is happening to cell volume, but whether the cells are shrinking or volume-regulating via cation/Cl^- uptake, we would have expected a large rise in intracellular chloride activity. This does indeed occur when the $Na^+ - HCO_3^-/Cl^-$ exchanger is blocked. The constancy of intracellular chloride is thus maintained by chloride exit via this exchanger. This exchanger could conceivably continue operating at control rates and still maintain a^i_{Cl} constant if chloride entry were to decrease in response to hypertonic solutions. The data suggest that the opposite occurs. If the cells behaved as perfect osmometers and no chloride movement occurred, a rise in intracellular chloride activity of 38% would be expected. In the HCO_3^--free and SITS experiments intracellular chloride activity increased by $86 \pm 14\%$ and $79 \pm 12\%$ (significantly greater than 38% in both cases with $P < 0.01$ and 0.025, respectively) during hypertonic perfusion, whereas in the Na^+-free experiment the increase was 40%. As intracellular chloride was already elevated due to the low Na^+ solution in this last group, the final a^i_{Cl} (and therefore the chemical gradient for chloride) after hypertonic perfusion in these studies was in the same range as in the bicarbonate-free and SITS studies despite the fact that the percentage increase was much less. It may be that this a^i_{Cl} represents the energetic limit for the entry pathway[1]. The fact that the rise in a^i_{Cl} is greater than predicted by osmotic considerations in some of the studies suggests that a chloride entry pathway has been stimulated by the hypertonic conditions. Therefore, it is likely that the exchanger-mediated chloride efflux could maintain a^i_{Cl} at a constant value only if the exchanger is effecting net outward chloride movement at a significantly faster rate than under control conditions.

We do not know the nature of the stimulated chloride entry pathway. Because only the peritubular solution was made hypertonic, solvent drag from lumen to cell could be responsible. We therefore cannot draw any conclusions regarding the presence or absence of volume regulatory pathways based on these studies. It is unlikely that the $Na^+ - HCO_3^-/Cl^-$ exchanger participates in volume regulation per se. The net osmotic result of its turnover is bicarbonate addition to the cell. Because of the high buffering capacity of the cell interior, a significant portion of this added bicarbonate will become CO_2 and water. Therefore only a fraction of the work of this exchanger will result in addition of osmotically active particles to the cell.

Whether these cells volume regulate or not, this exchanger does serve to regulate intracellular chloride activity under hypertonic conditions. Whether the regulatory stimulus is small changes in intracellular chloride concentration (or other ions carried by the exchanger) or whether an intracellular signal such as calcium or cyclic AMP is involved is not clear. With respect to the former possibility, a transient rise in chloride was never observed[2], but given the limits of the sensitivity of our methods, we could be missing a small change in a^i_{Cl} that could conceivably stimulate a sensitive system. On the other hand, calcium and cyclic AMP have been shown to stimulate the volume regulatory pathways in some cells [3, 11]. Though it is not clear whether these are the actual signals involved during volume changes, it is possible that this type of messenger can stimulate one type of pathway that regulates volume as well as other pathways that help regulate intracellular ion content during changes in tonicity. Further work needs to be done to determine the modulators of the kinetics of the $Na^+ - HCO_3^-/Cl^-$ exchange mechanism and to define its possible role in regulating intracellular chloride activity.

In summary, we have presented further evidence that the predominant mode of basolateral chloride exit in the *Necturus* proximal tubule is by way of a $Na^+ - HCO_3^-/Cl^-$ exchange pathway. The exchanger is stimulated when the tubule is exposed to hypertonic conditions resulting in a large chloride efflux and constancy of a^i_{Cl}.

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[1] If this entry process is electroneutral then the chemical gradient for chloride would be the relevant factor in considering energetics. If it were not neutral, the apical potential difference would be important and this was not measured. Furthermore, it is also possible that lowering basolateral sodium caused intracellular changes that affected this entry process.

[2] Presumably this was because the basolateral tonicity increased slowly in this experimental set-up so that the stimulated efflux was able to keep up with the forces tending to increase a^i_{Cl}.

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