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THE EFFECTS OF BICARBONATE AND HYDROXYL IONS ON CHLORIDE TRANSPORT BY TOAD BLADDERS

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Summary

The association between Cl^-, HCO_3^- and H^+ transport by toad bladders was investigated. Net mucosal to serosal Cl^- transport by Colombian toad bladders was stimulated by incubation in HCO_3^--free solutions. In addition, when Colombian or Dominican toad bladders were exposed to low HCO_3^- concentrations on the mucosal side and 25 mM HCO_3^- on the serosal side, net mucosal → serosal Cl^- transport was induced. Neither acetazolamide nor cyanide significantly inhibited Cl^- transport under these conditions. The presence of a pH gradient, more acid on the mucosal side, also induced net mucosal → serosal Cl^- transport. The results suggest that Cl^- transport by toad bladders may occur by exchange with HCO_3^- or OH^-; this process may not require carbonic anhydrase or oxidative metabolism. The Cl^- transport by toad bladders is qualitatively different from the electrogenic Cl^- transport of the thick limb of Henle's loop, but may be similar to a process which occurs in other portions of the nephron.

Introduction

A previous report from this laboratory [1] confirmed an initial report [2] that inhibition of Na^+ transport by the urinary bladder of Colombian toads caused a reversed short-circuit current and induced net Cl^- transport from the mucosal to serosal surface. In contrast to the initial report [2], removal of Cl^- from the mucosal bathing solution had very little effect on the reversed short-circuit current. It was thus concluded that Cl^- transport was not electrogenic. Furthermore, removal of HCO_3^- from bathing solutions eliminated the reversed short-circuit current, and the addition of acetazolamide inhibited both the reversed short-circuit current and net Cl^- transport. These findings suggested an association between the processes of Cl^-, H^+ [3], and/or HCO_3^- transport.

Net Cl^- transport occurs in a variety of tissues [2,4—12], but the mechanism of this process is unknown. Leslie and coworkers [13] suggested that Cl^--HCO_3^- exchange may occur in the urinary bladder of the fresh water turtle, as in other tissues [14—16]. The present experiments were designed to study the mechanism of active Cl^- transport in toad bladders by investigating the relationship between Cl^-, HCO_3^-, and H^+ transport. This work has been previously reported as abstracts [39—41].

Methods

Colombian toads (*Bufo marinus*) were purchased from Tarpon Zoo, Tarpon Springs, FL; and toads from the Dominican Republic (*B. marinus*) were obtained from National Reagents, Bridgeport, CT. The urinary hemibladders were removed and mounted between two glass chambers. Each side contained 15 ml of Ringer solution [1]. The cross-sectional area of the hemibladders exposed to the solution was 2.3 cm^2. The composition of the Ringer solutions used is shown in Table I. The osmolality of the solutions, determined with a Fiske osmometer, was 216—235 mosM/kg. Na^+ and K^+ were determined with an Instrumentation Laboratories Flame Photometer, and Cl^- was measured with an Aminco Chloridometer.

Electrical measurements were made with matched calomel half-cells connected to the bathing solutions with 0.11 M KCl/agar bridges. In the absence of bladders, the potential difference between the chambers was between +0.5 and —0.5 mV. With the bladders in place, a short-circuit current was applied with Ag|AgCl half-cells and KCl/agar bridges. Except during measurements of the potential difference between fluxes, the bladders were voltage clamped to maintain the potential difference at zero during the isotope flux experiments.

Unidirectional Cl^- fluxes were measured as previously described [1] by an adaptation of the methods of Finn et al. [2] and Ussing and Zerahn [17] using $Na^{36}Cl$ obtained from Amersham Searle. Initially, the isotope was added to the mucosal bathing solution. After an equilibration period of 30 min, 0.05 ml was removed from the mucosal (isotope-containing) side and 0.05 ml of solution

TABLE I

COMPOSITION OF INCUBATION SOLUTIONS

All Ringer solutions contained 5.5 mM glucose and 3.0 mM phosphate. Unmodified Ringer was gassed with 95% O_2/5% CO_2. All other Ringer was gassed with 100% O_2. Low bicarbonate Ringer solutions Nos. 2 and 3 were used in pH gradient experiments. KOH or HCl were added to adjust pH to either 6.0 or 7.5 (indicated in the tables). All data are in mM.

Type of solution	Na^+	K^+	Cl^-	HCO_3^-	Mg^{2+}	Ca^{2+}	SO_4	Methyl sulfate
Unmodified Ringer	114.2	2.5	88.2	25.0	1.2	1.6	1.8	0
Methyl sulfate Ringer	114.2	2.5	88.2	0	1.2	1.6	1.8	25.0
Low bicarbonate Ringer								
No. 1	113.2	2.5	112.2	0	1.2	1.6	1.8	6
No. 2	111.2	4.0	113.0	0	0	0	0	0
No. 3	111.2	5.4	113.0	0	0	0	0	0

was removed from the serosal side every 15 min for 90 min. Isotope-free Ringer solution was replaced to maintain a constant volume. At the end of the 90 min period, 0.05 ml was removed from the mucosal (isotope-containing) side. After each 90 min flux period, both chambers were repeatedly washed with isotope-free Ringer for at least 30 min in order to remove any isotope adhering to the bladder. Flux measurements were then repeated in the serosal to mucosal direction.

Radioactive samples were analyzed with a Nuclear Chicago Liquid Scintillation Counter. The Cl^- flux for each hemibladder was calculated as the mean of the six 15-min determinations. The '*n*' for each group of experiments is the number of hemibladders. All data are expressed as mean ± S.E. Student's *t*-test was used to determine statistical significance. When mucosal → serosal and serosal → mucosal fluxes were compared in the same hemibladders, the paired *t*-test was used to determine significance.

Results

Effects of bicarbonate on chloride transport (Table II)

In the previous report from this laboratory [1] it was found that removal of HCO_3^- from the solutions bathing Colombian toad bladders eliminated the reversed short-circuit current produced by prior removal of potassium from the bathing solution. However, measurements of Cl^- flux were not performed in those experiments.

Table II shows that Colombian toad bladders, incubated in unmodified Ringer solution, have no net Cl^- flux under short-circuited conditions. However, when the Colombian toad bladders are incubated in low HCO_3^- solutions, the mucosal → serosal Cl^- flux significantly increased from 0.27 ± 0.02 to

TABLE II

EFFECTS OF BICARBONATE ON CHLORIDE TRANSPORT

All data in this and subsequent tables are expressed as mean ± S.E. n.s., not significant; M, mucosal; S, serosal.

Conditions	Cl^- flux measurements (μmol/15 min)			Short-circuit current		
	M → S		S → M	Net M → S	μA	μmol./15 min
Colombian toads						
Unmodified Ringer	0.27		0.24	0.03	131	1.2
(*n* = 17)	(0.02)	*P* = n.s.	(0.03)	(0.02)	(19)	(0.2)
	P = <0.01		*P* = n.s.	*P* = <0.02	*P* = n.s.	
Low bicarbonate	0.52		0.29	0.23	119	1.1
Ringer No. 1	(0.09)	*P* < 0.01	(0.05)	(0.08)	(21)	(0.2)
(*n* = 17)						
Domican Republic toads						
Unmodified Ringer	0.34		0.33	0.01	61	0.6
(*n* = 11)	(0.07)	*P* = n.s.	(0.09)	(0.04)	(11)	(0.1)
	P = n.s.		*P* = n.s.	*P* = n.s.	*P* = n.s.	
Low bicarbonate	0.28	*P* = n.s.	0.36	0.08	56	0.5
Ringer No. 1	(0.06)		(0.08)	(0.08)	(12)	(0.1)

0.52 ± 0.09 μmol/15 min and the serosal → mucosal flux did not significantly change. Therefore, there was a significant net mucosal → serosal Cl^- flux of 0.23 ± 0.08 μmol/15 min under low HCO_3^- conditions. In contrast to all previous observations of net Cl^- transport in toad bladders, the short-circuit current was serosal positive instead of being serosal negative. Furthermore, the increase in net Cl^- flux from 0.03 ± 0.02 to 0.23 ± 0.08 μmol/15 min was associated with an insignificant change in the short-circuit current from 1.2 ± 0.2 to 1.1 ± 0.2 μmol/15 min (P = n.s.). This is consistent with the previous observations suggesting that Cl^- transport is not electrogenic.

Experiments were also performed with toads obtained from the Dominican Republic. Unlike Colombian toads, Dominican toads continue to be available for scientific investigation *. Bladders from Dominican toads do not generate a reversed short-circuit current and have been reported not to demonstrate active Cl^- transport [18,19]. When these bladders were incubated in unmodified or low HCO_3^- Ringer solutions, there was no significant net Cl^- transport.

Effects of bicarbonate gradients on chloride transport (Table III)

Under in vivo conditions, the serosal surface of the toad bladder is exposed to blood which contains HCO_3^-, while the mucosal surface is exposed to low HCO_3^- urine. To investigate the physiological relevance of Cl^- transport, experiments were performed under conditions designed to simulate the in vivo HCO_3^- gradient.

The upper portion of Table III demonstrates that, when Colombian toad bladders were incubated with 25 mM HCO_3^- on the serosal side and 0 mM HCO_3^- on the mucosal side, significant ($P < 0.001$) net Cl^- transport of 0.15 ± 0.03 μmol/15 min occurred. A reversal of HCO_3^- gradients was not associated with significant net Cl^- flux.

The lower portion of Table III depicts a similar experiment with Dominican Republic toads. When their bladders were incubated with 25 mM HCO_3^- on the serosal side and 0 mM HCO_3^- on the mucosal side, a small, but significant, net Cl^- flux of 0.05 ± 0.02 μmol/15 min ($P < 0.05$) was observed. This is the first demonstration of net Cl^- transport by Dominican Republic toad bladders.

Effects of acetazolamide and cyanide on chloride transport by Colombian toads in the presence of a bicarbonate gradient (Table IV)

Previous experiments demonstrated that acetazolamide inhibited the reversed short-circuit current and the net Cl^- flux produced by removal of potassium from the incubation solutions [1]. To investigate the role of carbonic anhydrase in Cl^- transport [13], the effects of acetazolamide [20—22] on chloride transport in the presence of a HCO_3^- gradient were studied (upper portion of Table IV). Both control and experimental observations were performed on each hemibladder. Under control conditions with a HCO_3^- gradient, there was a net mucosal → serosal flux of 0.22 ± 0.06 μmol Cl^-/15 min ($P < 0.02$). The addition of 10^{-3} M acetazolamide diminished the mucosal → serosal Cl^- flux from 0.51 ± 0.08 to 0.37 ± 0.05 μmol/15 min ($P < 0.02$), but

* Due to export restrictions by the Colombian government, Colombian toads are no longer available in the United States for scientific investigation.

TABLE III

EFFECTS OF BICARBONATE GRADIENTS ON CHLORIDE TRANSPORT

Unmodified Ringer solution (gassed with 95% O_2/5% CO_2) was used as the solution containing 25 mM bicarbonate. Methyl sulfate Ringer solution (gassed with 100% O_2) was used as the solution containing 0 mM bicarbonate. All solutions were pH 7.4. M, mucosal; S, serosal; n.s., not significant.

Conditions	Cl^- flux measurements (μmol/15 min)				Short-circuit current	
	M → S		S → M	Net M → S	μA	μmol/ 15 min
Colombian toads						
$M_{HCO_3^-}$ = 0 mM	0.32		0.17	0.15	117	1.1
$S_{HCO_3^-}$ = 25 mM	(0.03)	$P < 0.001$	(0.03)	(0.03)	(19)	(0.2)
(n = 14)						
$M_{HCO_3^-}$ = 25 mM	0.20		0.15	0.05	71	0.7
$S_{HCO_3^-}$ = 0 mM	(0.03)	P = n.s.	(0.03)	(0.03)	(9)	(0.1)
(n = 8)						
Dominican Republic toads						
$MHCO_3^-$ = 0 mM	0.28		0.22	0.05	189	1.8
$S_{HCO_3^-}$ = 25 mM	(0.05)	$P < 0.05$	(0.06)	(0.02)	(23)	(0.2)
(n = 11)						

TABLE IV

EFFECTS OF ACETAZOLAMIDE AND CYANIDE ON CHLORIDE TRANSPORT BY COLOMBIAN TOAD BLADDERS IN THE PRESENCE OF BICARBONATE

The bathing solutions were the same as in Table II. The measurements under control and inhibitory conditions were performed on the same hemibladder in a paired fashion. Acetazolamide and cyanide were added to both the mucosal and serosal solutions. After the addition of cyanide the short-circuit current rapidly fell. Subsequent additions of cyanide were sometimes necessary to keep the short-circuit current low. M, mucosal; S, serosal; n.s., not significant.

	Cl^- flux measurements (μmol/15 min)				Short-circuit current	
	M → S		S → M	Net M → S	μA	μmol/ 15 min
Effects of acetazolamide (n = 7)						
Control:						
$M_{HCO_3^-}$ = 0 mM	0.51		0.28	0.22	200	1.9
$S_{HCO_3^-}$ = 25 mM	(0.08)	$P < 0.02$	(0.04)	(0.06)	(26)	(0.2)
Experimental:	$P < 0.02$		$P < 0.02$	P = n.s.	P = n.s.	P = n.s.
$M_{HCO_3^-}$ = 0 mM	0.37		0.19	0.18	209	2.0
$S_{HCO_3^-}$ = 25 mM	(0.05)	$P < 0.01$	(0.02)	(0.04)	(37)	(0.4)
Acetazolamide (10^{-3} M)						
Effects of cyanide (n = 7)						
Control:						
$M_{HCO_3^-}$ = 0 mM	0.54		0.28	0.27	101	0.9
$S_{HCO_3^-}$ = 25 mM	(0.16)	$P < 0.01$	(0.10)	(0.08)	(10)	(0.02)
Experimental:	P = n.s.		P = n.s.	P = n.s.	$P < 0.001$	$P < 0.001$
$M_{HCO_3^-}$ = 0 mM	0.39		0.22	0.17	21	0.2
$S_{HCO_3^-}$ = 25 mM	(0.06)	$P < 0.05$	(0.07)	(0.04)	(2)	(0.02)
Cyanide (10^{-3} M)						

also significantly diminished serosal → mucosal flux ($P < 0.02$). Therefore, this high concentration of acetazolamide had no significant effect on net mucosal → serosal Cl^- transport.

To determine the role of cellular energy metabolism in transepithelial Cl^- transport, the effects of cyanide were investigated (lower portion of Table IV). The addition of 10^{-3} M cyanide significantly reduced the short-circuit current from 101 ± 10 to 21 ± 2 μA. However, cyanide had no significant effect on the mucosal → serosal Cl^- flux or net mucosal → serosal Cl^- transport.

The effects of pH gradients on chloride transport (Tables V and VI)

In addition to the HCO_3^- gradient which exists across the toad bladder in vivo, the presence of acid urine also creates a pH gradient across this epithelial tissue. Therefore, the effects of pH gradients on Cl^- transport by toad bladders were investigated. All these experiments were performed in HCO_3^--free solutions and acetazolamide was added to inhibit active Cl^- transport.

The upper portion of Table V demonstrates that in the presence of acetazolamide, there was no net Cl^- flux under low HCO_3^- conditions. However, when the mucosal bathing solutions were changed to pH 6.0, the mucosal → serosal Cl^- flux significantly increased from 0.26 ± 0.05 to 0.52 ± 0.13 μmol/15 min and a net mucosal → serosal Cl^- flux of 0.26 ± 0.08 μmol/15 min was measured. This was associated with a significant increase in the short-circuit current. The lower portion of Table V shows that the presence of 10^{-3} M KCN

TABLE V

EFFECTS OF pH GRADIENTS AND CYANIDE ON CHLORIDE TRANSPORT BY COLOMBIAN TOAD BLADDERS

All solutions contained 10^{-3} M acetazolamide. pH 7.5 buffer was low bicarbonate Ringer No. 3 and pH 6.0 buffer was low bicarbonate Ringer No. 2. The measurements under control and gradient conditions were performed on the same hemibladders in a paired fashion. M, mucosal; S, serosal; n.s., not significant.

	Cl^- flux measurements (μmol/15 min)				Short-circuit current	
	M → S		S → M	Net M → S	μA	μmol/15 min
Effects of pH gradient (n = 8)						
Control:						
M_{pH} = 7.5	0.26		0.021	0.05	132	1.2
S_{pH} = 7.5	(0.05)	P = n.s.	(0.04)	(0.02)	(2)	(0.01)
	$P < 0.01$		P = n.s.	$P < 0.01$	$P < 0.05$	$P < 0.05$
pH gradient						
M_{pH} = 6.0	0.52		0.27	0.26	188	1.8
S_{pH} = 7.5	(0.13)	$P < 0.01$	(0.06)	(0.08)	(31)	(0.3)
Effects of pH gradient and cyanide (n = 8)						
Control:						
M_{pH} = 7.5	0.32		0.33	0.01	85	0.8
S_{pH} = 7.5	(0.07)	P = n.s.	(0.06)	(0.02)	(19)	(0.2)
	$P < 0.01$		P = n.s.	$P < 0.001$	$P < 0.01$	$P < 0.01$
pH gradient and cyanide:						
M_{pH} = 6.0	0.68		0.34	0.34	21	0.2
S_{pH} = 7.5	(0.05)	$P < 0.01$	(0.05)	(0.08)	(6)	(0.1)

TABLE VI

EFFECTS OF pH GRADIENT ON CHLORIDE TRANSPORT BY DOMINICAN REPUBLIC TOAD BLADDERS

Solutions were the same as in Table IV. M, mucosal; S, serosal; n.s., not significant.

	Cl^- flux measurements (μmol/15 min)				Short-circuit current	
	M → S		S → M	Net M → S	μA	μmol/ 15 min
Control						
M_{pH} = 7.5	0.25		0.31	—0.06	60	0.6
	(0.05)	P = n.s.	(0.05)	(0.04)	(15)	(0.1)
S_{pH} = 7.5 (n = 9)						
	$P < 0.02$		P = n.s.	$P < 0.05$	P = n.s.	P = n.s.
pH gradient						
M_{pH} = 6.0	0.42		0.37	0.05	73	0.7
	(0.08)	P = n.s.	(0.08)	(0.04)	(18)	(0.2)
S_{pH} = 7.5 (n = 9)						

did not inhibit net Cl^- transport produced by a pH gradient, although the short-circuit current significantly fell from 85 ± 19 to 21 ± 6 μA.

Table VI shows the effects of a pH gradient on Cl^- transport by Dominican toad bladders. The presence of a mucosal pH of 6.0 significantly increased the mucosal → serosal Cl^- flux from 0.25 ± 0.05 to 0.42 ± 0.08 μmol/15 min ($P < 0.02$). The net mucosal → serosal Cl^- transport of 0.05 ± 0.04 μmol/15 min produced by the pH gradient was significantly greater than the net Cl^- transport of —0.06 ± 0.04 μmol/15 min determined under control conditions. Therefore, the imposition of a pH gradient significantly increased net mucosal → serosal Cl^- transport of Dominican toad bladders.

Discussion

The present investigation was designed to characterize the association between Cl^- and H^+ or HCO_3^- transport. In previous experiments, net Cl^- transport was stimulated by inhibition of Na^+ transport [1]. In the present study, net Cl^- transport by Colombian toad bladders was stimulated by alterations of HCO_3^- or H^+ concentrations in the bathing solutions, in the presence of active Na^+ transport.

Net Cl^- transport was found under several different circumstances. Table II shows that the absence of HCO_3^- in the incubation solutions stimulated Cl^- transport by Colombian, but not Dominican, toad bladders. Furthermore, a reversal of potential difference and short-circuit current did not occur in the absence of HCO_3^-; the potential difference remained serosal positive indicating that active Na^+ transport was continuing. However, the biological significance of these experiments is uncertain, because the toad bladder in situ is exposed to HCO_3^--containing blood on the serosal side.

The experiments shown in Table III were designed to simulate the HCO_3^- gradient which exists across the toad bladder in vivo. When Colombian toad

bladders were exposed to HCO_3^- gradients, a net mucosal → serosal Cl^- flux of 0.15 ± 0.03 μmol/15 min was observed. This occurred despite a serosal-positive short-circuit current of 117 ± 18 μA, suggesting active Na^+ transport from mucosal → serosal. When Dominican toad bladders were studied under the same conditions, a small, but significant net mucosal → serosal Cl^- flux of 0.05 ± 0.02 μmol/15 min was measured. Although previous investigators were unable to demonstrate net Cl^- transport by Dominican toad bladders after removal of serosal K^+ [18], the present results suggest that these bladders are capable of net Cl^- transport, when the incubation conditions resemble those found in situ.

It was previously reported by this laboratory [1] that when net Cl^- transport was stimulated by potassium removal, acetazolamide inhibited both the reversed short-circuit current and net Cl^- flux. To investigate further the role of carbonic anhydrase in Cl^- transport, the experiments with acetazolamide were performed (Table IV). In the presence of a HCO_3^- gradient. the addition of 10^{-3} M acetazolamide had no significant effect on net Cl^- flux. In addition, although 10^{-3} M cyanide reduced net Cl^- flux from 0.27 ± 0.08 to 0.17 ± 0.04 μmol/15 min, this decrease was not significant. It is concluded that neither carbonic anhydrase [20—23] nor the energy of oxidative metabolism is necessary for net Cl^- transport in the presence of a bicarbonate gradient. These findings are consistent with the hypothesis that net Cl^- transport occurs by exchange with bicarbonate [13,16]. If Cl^--HCO_3^- exchange does occur, the large serosal → mucosal HCO_3^- gradient would thus provide a chemical driving force for net mucosal → serosal Cl^- transport.

The mechanism by which inhibition of Na^+ transport stimulated net Cl^- transport [1] continues to be an enigma. If Cl^- is transported in exchange for HCO_3^-, it is not clear why inhibition of Na^+ transport should activate this process. Oliver and coworkers [24] have suggested that Cl^--HCO_3^- exchange is an energy-requiring process; it is possible that inhibition of Na^+ transport increases the availability of metabolic energy for the process of Cl^--HCO_3^- exchange [8,24]. This concept is not supported by the findings with cyanide (Table IV) which suggest that metabolic energy is not required for a portion of Cl^--HCO_3^- exchange. Therefore, Cl^- transport may not be truly active and may be coupled to the movement of other anions.

An alternative hypothesis is that Cl^- transport by toad bladders occurs in exchange with OH^-. Cl^- transport by mitochondria is normally electrogenic [25], but Cl^--OH^- exchange can be induced in these organelles [26—30]. Cl^--OH^- exchange has also been suggested to exist in intestine [31] and kidney proximal tubule [32]. The possibility of Cl^--OH^- exchange was examined by the experiments shown in Tables V and VI. A pH gradient (acid on the mucosal side) did induce net Cl^- transport by Colombian toad bladders in the presence of acetazolamide and cyanide (Table V). A similar pH gradient also stimulated the mucosal → serosal Cl^- transport by Dominican toad bladders (Table V). These findings suggest an explanation for the stimulation of net Cl^- transport by the inhibition of Na^+ transport. Inhibition of Na^+ transport diminishes the rate of cellular metabolism [33] which in turn should reduce metabolic acid production. This might cause a rise in the intracellular pH which would facilitate Cl^--OH^- exchange across the mucosal membrane. This hypothesis is highly

speculative and requires further experimental testing.

Prior to the present study, the physiological significance of net Cl^- transport by toad bladders was uncertain because it could only be demonstrated under conditions where Na^+ transport was inhibited. The present experiments show that HCO_3^- or pH gradients stimulate the Cl^- transport across the toad bladder, suggesting that the presence of these gradients in vivo may promote net Cl^- transport. Because net Cl^- transport under these conditions does not seem to directly require cellular energy, net Cl^- reabsorption promoted by the presence of HCO_3^- or pH gradients may be an efficient mechanism to maintain extracellular fluid volume.

The initial purpose of this project was to investigate the mechanism of active Cl^- transport by the thick ascending limb of Henle [5,10]. The findings that net Cl^- transport by toad bladders is non-electrogenic and is not inhibited by ouabain [1] suggest that it is a qualitatively different process than that which exists in the thick ascending limb. However, the toad bladder seems to be more closely analogous to the distal or collecting tubules of the mammalian kidney, and active Cl^- transport has also been suggested to occur in these nephron segments [34—38]. The process of active H^+ secretion by the distal and collecting tubules results in HCO_3^- and pH gradients; it is therefore possible that some fraction of Cl^- reabsorption in the distal portion of the nephron may occur by a process similar to that observed in the toad urinary bladder.

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