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Electrogenic Cl^- Absorption by *Amphiuma* Small Intestine: Dependence on Serosal Na^+ from Tracer and Cl^- Microelectrode Studies

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Summary. The Na^+ requirement for active, electrogenic Cl^- absorption by *Amphiuma* small intestine was studied by tracer techniques and double-barreled Cl^--sensitive microelectrodes. Addition of Cl^- to a Cl^--free medium bathing *in vitro* intestinal segments produced a saturable ($K_m = 5.4$ mM) increase in short-circuit current (I_{sc}) which was inhibitable by 1 mM SITS. The selectivity sequence for the anion-evoked current was $Cl^- = Br^- > SCN^- > NO_3^- > F^- = I^-$. Current evoked by Cl^- reached a maximum with increasing medium Na concentration ($K_m =$ 12.4 mM). Addition of Na^+, as Na gluconate (10 mM), to mucosal and serosal Na^+-free media stimulated the Cl^- current and simultaneously increased the absorptive Cl^- flux ($J^{Cl}_{m\to s}$) and net flux (J^{Cl}_{net}) without changing the secretory Cl^- flux ($J^{Cl}_{s\to m}$). Addition of Na^+ only to the serosal fluid stimulated $J^{Cl}_{m\to s}$ much more than Na^+ addition only to the mucosal fluid in paired tissues. Serosal DIDS (1 mM) blocked the stimulation. Serosal 10 mM Tris gluconate or choline gluconate failed to stimulate $J^{Cl}_{m\to s}$. Intracellular Cl^- activity (a^i_{Cl}) in villus epithelial cells was above electrochemical equilibrium indicating active Cl^- uptake. Ouabain (1 mM) eliminated Cl^- accumulation and reduced the mucosal membrane potential (ψ_m) over 2 to 3 hr. In contrast, SITS had no effect on Cl^- accumulation and hyperpolarized the mucosal membrane. Replacement of serosal Na^+ with choline eliminated Cl^- accumulation while replacement of mucosal Na^+ had no effect. In conclusion by two independent methods active electrogenic Cl^- absorption depends on serosal rather than mucosal Na^+. It is concluded that Cl^- enters the cell via a primary (rheogenic) transport mechanism. At the serosal membrane the Na^+ gradient most likely energizes H^+ export and regulates mucosal Cl^- accumulation perhaps by influencing cell pH or HCO_3^- concentration.

Key Words chloride · sodium · intestine · *Amphiuma* · Cl^--sensitive microelectrodes · intracellular Cl^- activity · bicarbonate

Introduction

Active Cl^- absorption observed in many epithelia commonly exhibits an absolute dependence on Na^+. However, the exact role of Na^+ is unclear with evidence that Na^+ and Cl^- are cotransported simultaneously [5, 11, 12, 17–20] contrasting with evidence that separate, parallel Na^+/H^+ and Cl^-/HCO_3^- exchanges underlie Cl^- transport [6, 7, 15, 16]. Indeed it has been suggested that both types of transport (symport and antiport) exist simultaneously [7, 9]. By either route Cl^- transport would be electrically silent. On the other hand, in certain epithelia net Cl^- absorption is electrogenic [2, 8, 23]. For example, Cl^- absorption generates a serosa negative electrical potential difference in *Amphiuma* small intestine even though net Cl^- absorption is absolutely dependent on the presence of Na^+ and HCO_3^- in the bathing medium and is accompanied by HCO_3^- secretion. We have demonstrated with Cl^--selective microelectrodes that Cl^- is actively accumulated by the villus absorptive cells of *Amphiuma* and that this accumulation is eliminated when Na^+ is replaced in both the mucosal and serosal fluids bathing the mucosa [23]. This observation is suggestive of the presence of luminal Na-Cl cotransport. However, we reported more recently that the ability of Cl^- added to the luminal fluid to elicit active HCO_3^- secretion requires Na^+ not in the mucosal but rather in the serosal bathing fluid [26]. It was concluded that since the Cl^-/HCO_3^- exchange process occurs in the basolateral membrane, beyond the active Cl^- uptake step at the brush border membrane, then Na^+ must not play a direct role in active intracellular Cl^- accumulation. In addition to suggesting that active Cl^- uptake is electrogenic, the results pointed to important new experimental approaches which would provide a more definitive test of our hypothesis. In the present paper we describe two new lines of evidence for a role of serosal Na^+ rather than mucosal Na^+ in active electrogenic Cl^- absorption. For this, isotope flux measurements and double-barreled Cl^--selective microelectrodes were employed.

Materials and Methods

Animal and Tissue Preparation

Adult *Amphiuma* were maintained and anesthetized as described previously [22]. Duodenal or jejunal segments were ex-

Table 1. Composition of bathing media

	Normal media	Na^+-free media	Cl^--free media	$Na^+ - Cl^-$-free media
Na^+	95	0	95	0
$Choline^+$	0	95	0	95
Cl^-	76.3	76.3	0	0
SO_4^{2-}	0	2.25	37.3	37.3
HCO_3^-	25	25	25	25
K^+	2.5	2.5	2.5	2.5
Ca^{2+}	0.9	0.9	0.9	0.9
Mg^{2+}	1.0	1.0	1.0	1.0
Gluconate	0	0	1.8	1.8
Mannitol	20	20	56.5	56.5

cised and stripped of their outer muscle layers while bathed in oxygenated media. For the current and isotope flux measurements two adjacent intestinal segments were each opened and mounted as a sheet in separate Plexiglass® chambers.

Bathing Media

The bathing media employed are listed in Table 1. Na^+ was replaced with choline and Cl^- with So_4^{2-}. All solutions contained 25 meq/liter HCO_3^- and were gassed with 95% O_2/5% CO_2. The choline sulfate employed in the $Na^+ + Cl^-$-free media was prepared by titration of choline bicarbonate to pH 7.4 with H_2SO_4. Choline gluconate was prepared by titrating gluconic acid with choline base. Tris gluconate was prepared by titrating tris hydroxymethyl aminomethane with gluconic acid. Bathing media were prepared from stock solutions stored under refrigeration. The media were also stored in the cold (maximally 2 to 3 days) until use. The decomposition of choline-based solutions was minimal (e.g. refrigerated stock solutions of choline bicarbonate did not change in osmolality over a two-week period). The osmolality of bathing media were checked routinely with a Wescor 5,100 C vapor pressure osmometer. When Cl^- was substituted with SO_4^{2-}, the free Ca^{++} in solution was 0.4 to 0.5 mM. Additional studies performed using media with normal [Ca^{++}] gave results indistinguishable from those employing the low Ca^{++} media. All chemicals were reagent grade. SITS (4-acetamido-4′-isothiocyanostilbene-2-2′-disulfonate) and DIDS (4,4′-diisothiocyano-2,2′-stilbene disulfonate) were purchased from Polysciences (Warrington, Pa.). Ouabain was obtained from Sigma (St. Louis, Mo.).

Experimental Procedure

Intestinal segments were placed in Lucite® chambers suitable for measurement of either short-circuit current or intracellular Cl^- activity. For the former purpose two identical Ussing-type tissue chambers described previously [23] were assembled and pre-equilibrated with the appropriate bathing medium for 1 hr before the tissue was inserted. The short-circuit current (I_{sc}) was measured with a voltage-clamp device which compensated automatically for the resistance of the bathing medium employed during the pre-equilibration period [25]. When the [Na^+] of the medium was varied, the potential-sensing and current-passing agar bridges were formed with saturated KCl and Na^+-free media, respectively; when the [Cl^-] was varied the bridges were made with 1 M Na formate and Cl^--free media, respectively. When both Na^+ and Cl^- were replaced all agar bridges were formed with choline SO_4^{2-} media. After the tissue was in place both compartments of the tissue chamber were rinsed repeatedly with fresh medium. This was done for 1 hr when ion replacements were performed. Otherwise a 20-min rinse was employed.

Isotope Fluxes

Unidirectional mucosal to serosal ($m \rightarrow s$) and serosal to mucosal ($s \rightarrow m$) fluxes of Na and Cl were measured with ^{22}Na (as NaCl) and ^{36}Cl (as HCl) purchased from New England Nuclear. The latter was titrated with Tris base to pH 7.3. The flux measurement has been described in detail previously [23]. Briefly, the isotopes were added to the appropriate medium to 1 μCi/ml and 1 hr later a 100-μl sample was withdrawn from the opposite compartment. The volume of the source compartment was maintained by subsequent addition of 100 μl of fresh bathing medium. Four samples were usually collected at 20-min intervals, providing three flux periods. Na gluconate or another salt was then added to mucosal, serosal, or both compartments and, after a new steady-state I_{sc} had developed (usually 60 min), four more samples were collected at 20-min intervals. Specific activity was determined from triplicate samples of the hot side. The reported ion fluxes, in μeq/hr·cm^2, are the mean of the average of the three flux periods.

Cl^- Sensitive Microelectrodes

These were prepared in a double-barreled configuration, one barrel sensing Cl^- activity, the other membrane potential. A different method of fabrication was employed than used previously [22, 23]. Pyrex® capillary tubes were cleaned, first in a soap solution, then rinsed, boiled in absolute alcohol, dried and stored in the oven. A length of Kwik-Fil® capillary glass (WPI, New Haven, Conn.) was attached side-by-side to a longer length of Pyrex capillary glass using heat shrinkable tubing. One end of the double-barreled capillary was secured into a chuck and the other into the lower grip of a Kopf microelectrode puller (Tujunga, Calif.). After heating, the double-barreled capillary was rotated 270° and allowed to pull. The longer barrel was exposed through the open end for 60 to 100 sec to vapors of dimethyl dicholorosilane, placed tip up into a metal holder and baked at 250 °C for 1 hr. A column of Corning Cl^--sensitive liquid ion exchanger #477913 (Corning Medical, Medfield, Mass.) was introduced into the rear of the silanized barrel by means of a syringe. With the aid of a fine glass fiber and a microforge the exchanger was drawn to the tip of the barrel. Behind the exchanger 0.5 M KCl was placed. The Kwik-Fil barrel was then filled with 1 M Na formate. The tip diameter of the final double-barreled microelectrode (DBM) was less than 1 μm.

Each barrel of the DBM was connected via chloridized Ag wire to its respective amplifier. The voltage on the chloride-sensing barrel, ψ_{Cl}, was sensed by a high-input impedance (10^{15} Ω) electrometer (WPI Model FD 223) while the voltage on the membrane potential-sensing barrel, ψ_m, was sensed by a WPI model M 701 amplifier. Both voltages were recorded relative to a grounded reference electrode in the mucosal bath on separate channels of a Gould Brush recorder. In addition, the difference in voltage between the two barrels, $\psi_{Cl} - \psi_m$, was recorded on a third channel. For 12 electrodes the resistance of the Cl^--sensing barrel was $29 \pm 5 \times 10^9$ Ω; for the membrane-potential sensing barrel 43 ± 8 MΩ. The double-barreled microelectrodes were routinely calibrated in solutions of 10, 20 and 100 mM NaCl and in 100 mM $NaHCO_3^-$. The slope (S) of the

electrode response was calculated from the regression line of voltage *vs.* the log of the Cl^- activity and averaged -52.7 ± 0.4 mV in 88 DBM. The intracellular chloride activity (a^i_{Cl}) was calculated using Eq. (1):

$$a^i_{Cl} = a''_{Cl}/e^{2.303\ (\psi^i_{Cl} - \psi_m) - \psi''_{Cl}/S} \quad (1)$$

where a''_{Cl} and ψ''_{Cl} are the chloride activity and potential of the 100 mM NaCl standard and ψ^i_{Cl} is the potential of the Cl^- barrel in the cell. The equilibrium potential (E_{Cl}) for Cl^- at the mucosal membrane was calculated from Eq. (2):

$$E_{Cl} = \frac{RT}{F} \ln \quad a^i_{Cl}/a^o_{Cl} \quad (2)$$

where $\frac{RT}{F}$ equals 25.4 mV at room temperature (22 °C), and a^o_{Cl} is the bath Cl^- activity (60.7 mM). Bath Cl^- activity measured independently with a Ag/AgCl electrode was 59.1 ± 1.0 mM. The sensitivity for Cl^- over HCO_3^- was 10.1:1. The exchanger was unaffected by choline and responded poorly to SITS as well. For example, the change in voltage between normal medium and medium which contained 1 mM SITS was -2.4 ± 0.5 mV in 8 measurements. We have previously observed a greater responsiveness to SITS in microelectrodes containing Corning Cl^- exchanger #477315 silanized by an earlier method [22] which employed 1% Siliclad® in 1-chloronaphthalene (*unpublished observations*).

For Cl^- activity measurements a Lucite® tissue chamber of new design was used which had a configuration of pins which allowed maximal tension to be placed on the intestine before the segment was clamped between the mucosal and serosal chamber halves. This essentially eliminated all spontaneous smooth muscle activity. Each half-chamber was perfused with medium from separate reservoirs. At the flow rate employed the mucosal volume (0.6 ml) was replaced 6 times per minute. The transepithelial potential was sensed by a voltage-clamp device through calomel cells connected to the chamber via saturated. KCl-agar bridges. The voltage was displayed on the fourth channel of the Brush recorder. The DBM were advanced with a hydraulic microdrive (Kopf Inst., Tujunga, Calif.). Using the same DBM, measurements were made in several villus cells prior to calibration. Those recordings were deemed acceptable when i) the difference in potential between the two barrels while in the cell ($\psi^i_{Cl} - \psi_m$) was constant within 1 mV for 10 sec, ii) the tip potential of the barrel sensing ψ_m did not change more than 3 mV from penetration to withdrawal and iii) the Cl^- exchanger responded to changes in a_{Cl} with a slope S greater than -45 mV.

Statistical Tests

Student's *t*-test for paired or unpaired comparisons were used to determine the significance of the difference between population means.

Results

Isotope Flux Studies

Electrogenic Cl^- Absorption

Addition of increasing amounts of Cl^- to a Cl^--free medium bathing *Amphiuma* small intestine caused a saturable increase in I_{sc} consistent with greater Cl^- absorption. In Fig. 1 a typical response is illustrated wherein two adjacent intestinal segments, one pre-exposed on the serosal surface to the disulfonic stilbene SITS (1 mM), were exposed to increasing concentrations of Cl^- added as choline chloride simultaneously to mucosal and serosal media. On average for four pairs of tissues there was greater stimulation of the current with greater concentrations of Cl^- in the bathing medium. The response was completely blocked by SITS. As seen in Fig. 2*a* the stimulation of current approached a maximum. Lineweaver-Burk analysis (Fig. 2*b*) revealed an apparent $K_m = 5.4$ mM for Cl^- and suggested that SITS is a noncompetitive inhibitor of the response (reduced V_{max}, increased K_m). It was previously shown that SITS nearly completely blocks active Cl^- absorption in *Amphiuma* small intestine [27, 27].

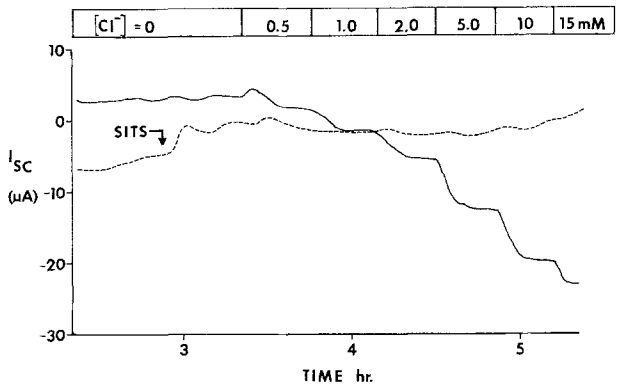

Fig. 1. Response of paired segments to increasing concentrations of Cl^-. Tissues were incubated initially in a Cl^--free (SO_4^{2-}-based) medium. SITS (1 mM) was added at the arrow to the serosal medium of one segment (dashed line). The small change in I_{sc} after SITS addition is most likely due to inhibition of electrogenic HCO_3^- absorption which occurs in this tissue [1, 2]. Cl^- was added as choline chloride in small volumes (30 μl) of a concentrated stock (1.7 M)

Of several anions tested only Br^- was able to produce comparable stimulation of the I_{sc}. In Fig. 3 it is seen that the stimulation of the I_{sc} varied greatly after addition of the Na salt of several anions to a final concentration of 5 mM in separate tissues. Assuming the anions are binding to a specific transport site of the epithelial cell then the results suggest a selectivity sequence $Cl^- = Br^- > SCN^- > NO_3^- > F^- = I^-$. This sequence is nearly identical to the sequences observed for anion efflux rate constants from red cells [3, 21] and points to the presence of anion binding sites of high field strength [28]. The selectivity sequence for anion influx across the brush border membrane of rabbit intestinal epithelium is very different [10] and corresponds to anion binding sites of low field strength [28].

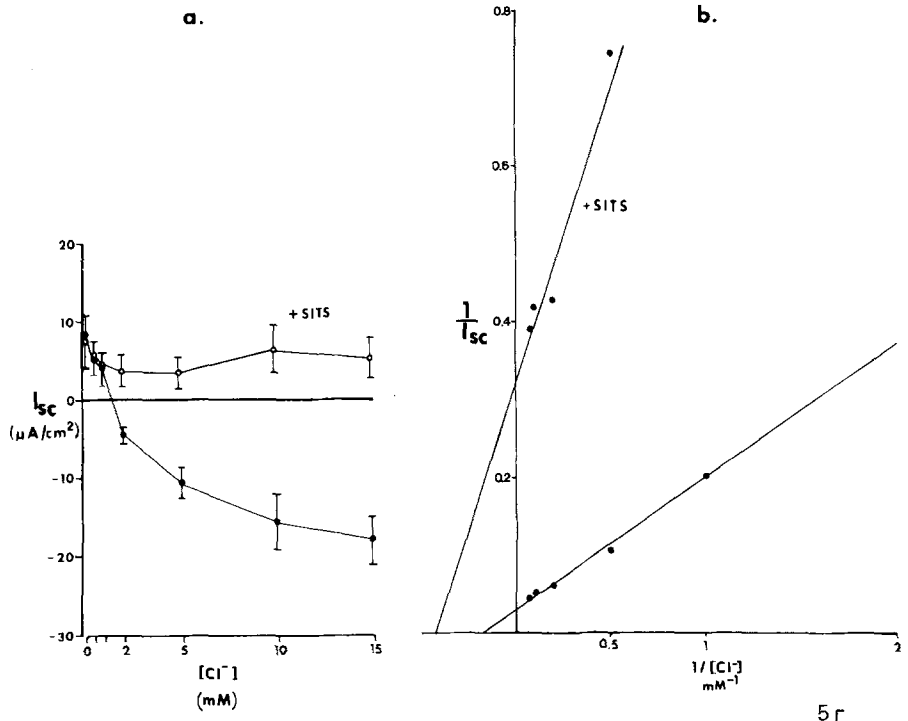

Fig. 2. (*a*) Average response of 4 paired segments subjected to the protocol in Fig. 1. Lines were drawn by eye. (*b*) Double-reciprocal plot of data points. The points at low medium Cl^-, which are of least accuracy, were disregarded. SITS increased the K_m and lowered the V_{max}

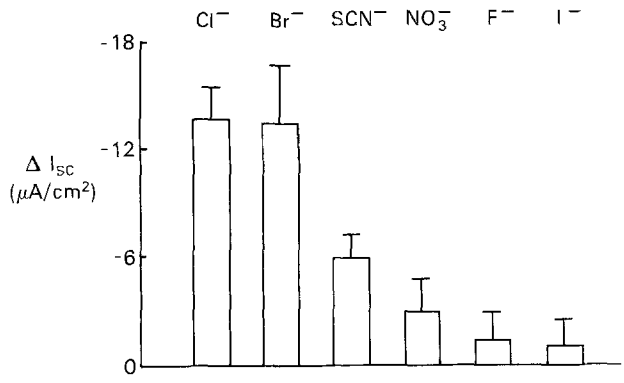

Fig. 3. Average increase in I_{sc} following addition of anion as Na salt to 5 mM final concentration in mucosal and serosal medium. Each point is the average of 3 or 4 observations in unpaired tissues

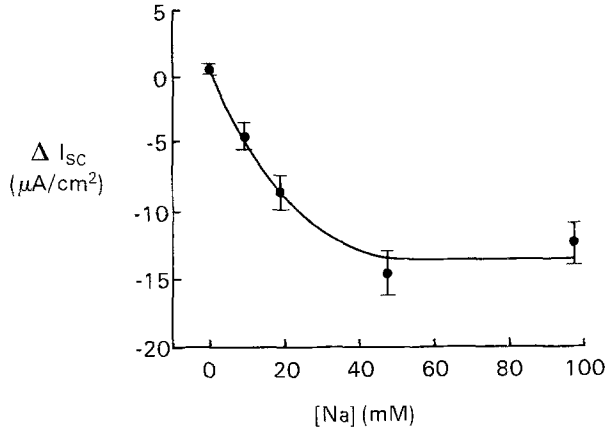

Fig. 4. Stimulation of I_{sc} by Cl^- as a function of medium $[Na^+]$. Cl^- was added (as choline Cl^-) to 10 mM on both sides. Each point is the average of 5 to 8 observations

Dependence on Na^+

In Fig. 4 the response of the I_{sc} to addition of 10 mM choline Cl to both media is seen to be a saturating function of the [Na] of the medium. When Na^+ was completely replaced with choline then addition of Cl^- failed to stimulate the I_{sc}. With increasing amounts of Na^+ in the medium the I_{sc} was increased, saturating when [Na] = 50 mM. The apparent K_m was 12.4 mM Na. In this tissue net Cl^- absorption is abolished by complete replacement of medium Na^+ [23].

The results in Fig. 4 suggested that addition of Na^+ to a Na^+-free medium could stimulate electrogenic Cl^- absorption and the I_{sc}. As seen in Fig. 5 addition of Na^+ as sodium gluconate (Na Gluc) simultaneously to Na-free mucosal and serosal media increased the I_{sc} to a new steady state consistent with greater electrogenic Cl^- absorption. The final $[Na^+]$ was 10 meq/liter. In Table 2 it is seen that Na^+ addition caused a highly significant ($P<0.001$) increase in the absorptive flux ($J^{Cl}_{m\to s}$) of radiolabeled Cl (Cl^{36}) without stimulating the secretory Cl^- flux ($P>0.50$). Net Cl^- flux was stimulated significantly ($P<0.01$) by 0.42 ± 0.08 $\mu eq/hr\cdot cm^2$. Simultaneously there was a smaller increase in I_{sc} of 0.21 ± 0.03 $\mu eq/hr\cdot cm^2$. The difference in the two values is most likely ac-

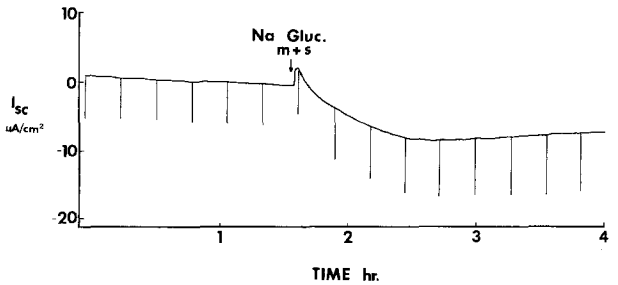

Fig. 5. Stimulation of current after addition of small volume of concentrated sodium gluconate (NaGluc) to a final concentration of 10 mM in mucosal (*m*) and serosal (*s*) solution. Medium was initially Na^+-free. The intermittent shift in the trace is due to periodic change in external current passage to allow estimation of tissue resistance. Na gluconate lowered the tissue resistance

Table 2. Effect of Na^+ on unidirectional Cl^- fluxes[a]

	period 1: control	period 2: + Na Gluc		*P*
	J^{Cl} (μeq/hr·cm²)	J^{Cl} (μeq/hr·cm²)	ΔJ^{Cl} (μeq/hr·cm²)	
a) Na^+ addition, $m+s$ ($n=5$)				
$m \rightarrow s$	0.79±0.03	1.21±0.04	0.43±0.04	<0.001
$s \rightarrow m$	0.75±0.04	0.76±0.10	0.01±0.08	N.S.[b]
			ΔJ^{Cl}_{net}=0.42±0.08	<0.01
b) Na^+ addition to *s* ($n=4$)				
$m \rightarrow s$	0.83±0.06	1.29±0.08	0.46±0.05	<0.01
$s \rightarrow m$	0.91±0.10	0.97±0.12	0.06±0.05	N.S.
			ΔJ^{Cl}_{net}=0.40±0.10	<0.01
c) Na^+ addition to *m* ($n=4$)				
$m \rightarrow s$	0.92±0.13	122±0.16	0.31±0.05	<0.01
$s \rightarrow m$	0.90±0.13	0.95±0.14	0.05±0.03	N.S.
			ΔJ^{Cl}_{net}=0.26±0.07	<0.05

[a] Sodium gluconate (Na Gluc) was added to a final concentration of 10 mM.
[b] N.S. = not significant at $P=0.05$.

counted for by an enhanced parallel secretory anion current carried by HCO_3^-. In *Amphiuma* small intestine Cl^- absorption is absolutely dependent on HCO_3^- in the medium and is associated with a HCO_3^- secretory flux [14, 23].

Sidedness of Na^+ Dependence: Unpaired Studies

Addition of Na^+ only to the serosal medium also increased the Cl^- absorptive flux. In Table 2 it is seen that $J^{Cl}_{m\rightarrow s}$ was increased by 0.46±0.05 μeq/hr·cm². In contrast in unpaired tissues, addition of Na^+ only to the mucosal medium produced a 1/3 rd smaller stimulation of the Cl^- absorptive

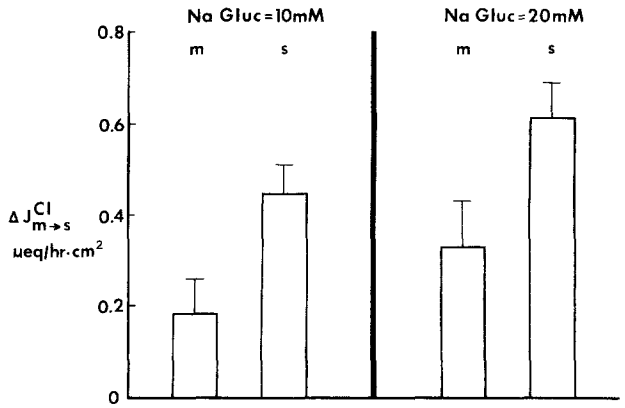

Fig. 6. Stimulation of the Cl^- absorptive flux ($\Delta J^{Cl}_{m\rightarrow s}$) by NaGluc added to *m* or s *in* paired tissues. Left: Final Na gluconate concentration = 10 mM, $n=5$. Right: Final Na gluconate concentration = 20 mM, $n=5$

flux. Comparison of the fluxes reveals that addition of Na^+ solely to the serosal medium was sufficient to give all of the response produced by Na^+ added to both media. However, the stimulation of $J^{Cl}_{m\rightarrow s}$ after mucosal addition of Na^+, although lower, was not significantly ($P>0.10$) less than after serosal addition. This point was re-examined in paired tissues.

Sidedness of Na^+ Dependency: Paired Tissues

As illustrated in Fig. 6 (left), when the stimulation of the Cl^- absorptive flux ($\Delta J^{Cl}_{m\rightarrow s}$) due to mucosal addition of 10 mM Na^+ was compared to the stimulation upon serosal Na addition in paired adjacent segments, there was a significantly ($P<0.01$) greater enhancement due to serosal Na^+. Na^+ addition to the serosal medium was sufficient to provide maximal stimulation of Cl^- absorption, whereas stimulation by Na^+ added to the mucosal medium was submaximal and most likely due to Na^+ entry into the serosal fluid bathing the basolateral membrane by way of the paracellular path.

The dependence on serosal Na^+ was also observed after addition of Na^+ to a higher concentration (20 mM) in another series. As seen in Fig. 6 (right) addition of Na^+ to the serosal medium to 20 mM caused greater stimulation of $J^{Cl}_{m\rightarrow s}$ than did 10 mM Na^+, and the response was greater than upon mucosal addition of Na^+ in paired tissues ($P<0.05$). Furthermore, the response following mucosal addition to 20 mM exceeded that due to 10 mM Na^+. These results further illustrate the dependence of Cl^- absorption on Na^+ and specifically serosal Na^+, and are consistent with the view that mucosally added Na^+ stimulates $J^{Cl}_{m\rightarrow s}$ by gaining access to the serosal fluid.

Table 3. Effect of SITS on Na^+-stimulated Cl^- absorption[a]

	$J^{Cl}_{m\to s}$ (μeq/hr·cm^2)		$\Delta J^{Cl}_{m\to s}$	P
	no Na^+	20 mM Na^+ Gluc		
control	0.81±0.03	1.47±0.11	0.66±0.10	<0.01
+SITS	0.52±0.05	0.57±0.06	0.06±0.03	N.S.

[a] SITS was present at 1 mM ($n=5$)

Table 4. Effect of cations on Cl^- absorption

Series	n		$\Delta J^{Cl}_{m\to s}$ (μeq/hr·cm^2)	P
1	(4)	+Na Gluc	0.32±0.07	<0.05
		+Choline Gluc	0.02±0.11	N.S.
2	(5)	+Na Gluc	0.32±0.03	<0.01
		+Tris Gluc	0.01±0.05	N.S.

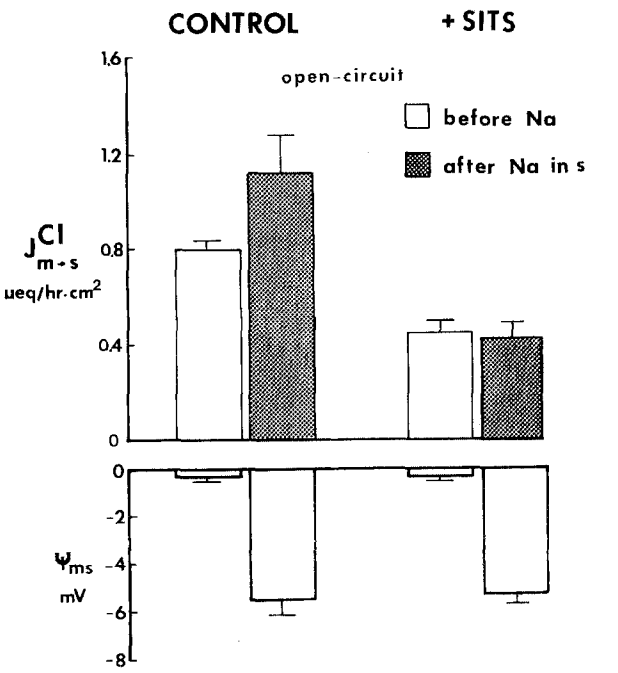

Fig. 7. Stimulation of Cl^- absorptive flux by serosal Na^+ in open-circuited tissues. Transepithelial potential (ψ_{ms}) was serosa negative with respect to mucosa. Tissues were paired, one was the control, the other exposed to SITS. Final Na concentration = 10 mM, $n=5$

Effect of SITS on Na-Stimulated $J^{Cl}_{m\to s}$

As seen in Table 3, the disulfonic stilbene SITS blocked the stimulation of $J^{Cl}_{m\to s}$ by serosal Na^+. In control segments, 20 mM Na^+ increased $J^{Cl}_{m\to s}$ by 0.66±0.10 μeq/hr·cm^2 ($P<0.01$), nearly identical to the previous series (Table 2). In paired intestines exposed on their serosal surface to 1 mM SITS, there was no significant stimulation ($P>0.10$). Indeed, the value of $J^{Cl}_{m\to s}$ in SITS-treated tissues was significantly reduced ($P<0.001$) from controls even before Na^+ addition, suggesting that the Na^+-independent (passive) absorptive flux of Cl^- occurs partly over the transcellular stilbene-sensitive pathway.

Since SITS completely blocked the Na^+-stimulated Cl^- flux, it was possible to differentiate the increase in I_{sc} due to electrogenic Cl^- absorption from the increase due to the imposition of an electrodiffusional flux of Na^+ that occurs when Na^+ is added unilaterally to the mucosal medium. Thus in the absence of SITS the I_{sc} was 1.05±0.09 μeq/hr·cm^2 while in its presence I_{sc} was 0.61±0.03 μeq/hr·cm^2. The difference, 0.44±0.08 μeq/hr·cm^2 was highly significant ($P<0.01$) and attributable to electrogenic Cl^- absorption. However, since $\Delta J^{Cl}_{m\to s}$ was higher at 0.60±0.11 μeq/hr·cm^2 when measured isotopically, then another ion movement such as the secretion of HCO_3^- must occur simultaneously.

Specificity of Na^+ Dependence

Neither choline nor Tris were able to stimulate Cl^- absorption. The two series in Table 4 show that addition of Na gluconate to a final concentration of 10 mM stimulated $J^{Cl}_{m\to s}$ while in paired intestinal segments addition of either choline gluconate or Tris gluconate had no effect. In addition to demonstrating the specificity of stimulation by Na^+ this result also rules out the possibility that serosal addition of salt stimulates $J^{Cl}_{m\to s}$ indirectly through an effect on medium osmolarity.

Stimulation by Na^+ in Open-Circuit

The stimulating effect of serosal Na^+ was also evident in open-circuited preparations. As seen in Fig. 7, $J^{Cl}_{m\to s}$ was elevated after addition of Na gluconate to the serosal medium in the face of an increased serosa negative transepithelial electrical potential difference resulting from electrodiffusion of Na^+. SITS blocked the stimulation of $J^{Cl}_{m\to s}$ in paired tissues indicating that the Cl^- flux was transcellular.

Effect of Cl^- on Na^+ Fluxes

In Table 5 it is seen that addition of Cl^- (as choline chloride) simultaneously to mucosal and serosal Cl^--free media stimulated the I_{sc} but had no significant effect ($P>0.40$) on the unidirectional fluxes of radiolabeled Na^+. Thus, while addition of Na^+ is able to stimulate Cl^- absorption, addition of Cl^- is not able to stimulate Na^+ absorption in a like manner.

Table 5. Effect of Cl^- on unidirectional Na^+ fluxes[a]

	$J^{Na}_{m\to s}$	$J^{Na}_{s\to m}$	I_{sc}
Control	3.36±0.2	3.93±0.49	−0.03±0.02
10 mM Cl^-	3.57±0.3	4.11±0.59	−0.61±0.08
Δ	0.21±0.23	0.17±0.18	−0.58±0.08
P	N.S.	N.S.	<0.001

[a] Rates are μeq/hr·cm². Cl^- was added as choline chloride to a final concentration of 10 mM. The I_{sc} is the average for six pairs of tissues.

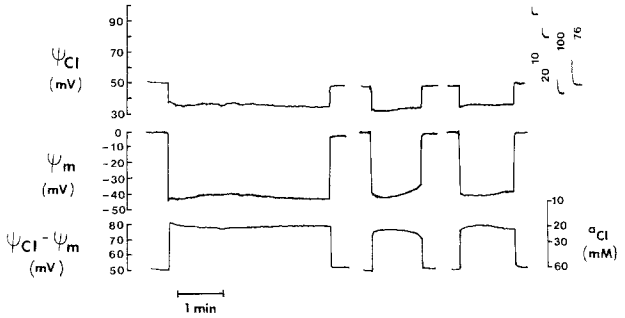

Fig. 8. Three separate recordings of intracellular Cl^- activity using double-barreled microelectrodes. The potential of the Cl^--sensing barrel (ψ_{Cl}) and the membrane potential-sensing barrel (ψ_m) when subtracted provided a voltage related to chloride activity (a_{Cl}). Calibration in solutions of different Cl^- concentration (10, 20, 76 and 100 mM) is also illustrated

Cl^--Sensitive Microelectrode Measurements

Intracellular Cl^- Activity

Cl^- is actively accumulated by the epithelial cells lining the villus of the small intestine in common with other intestinal preparations [1, 4]. As seen in Fig. 8, when double-barreled microelectrodes were driven across the luminal membrane there were changes in the potential of the Cl^--sensitive barrel (ψ_{Cl}), the membrane potential-sensing barrel (ψ_m) and the difference between the voltages ($\psi_{Cl}-\psi_m$). The latter is related to intracellular Cl^- activity (a^i_{Cl}) as indicated in the Figure. Between periods of calibration penetrations were made into several villus cells each located several hundred microns from the other along a villus. The stability of the voltages was quite variable; in some cases the voltages were maintained several minutes. More frequently there was a tendency for the voltages to decay. Only those recordings which met specified criteria (*see* Materials and Methods) were analyzed further. From Eqs. (1) and (2), a^i_{Cl} and E_{Cl}, the electrochemical gradient for Cl^-, were calculated. In agreement with previous studies [21, 22], Cl^- was actively accumulated (i.e. $E_{Cl}>\psi_m$) by 10 to 15 mV by *Amphiuma* intestinal absorptive cells.

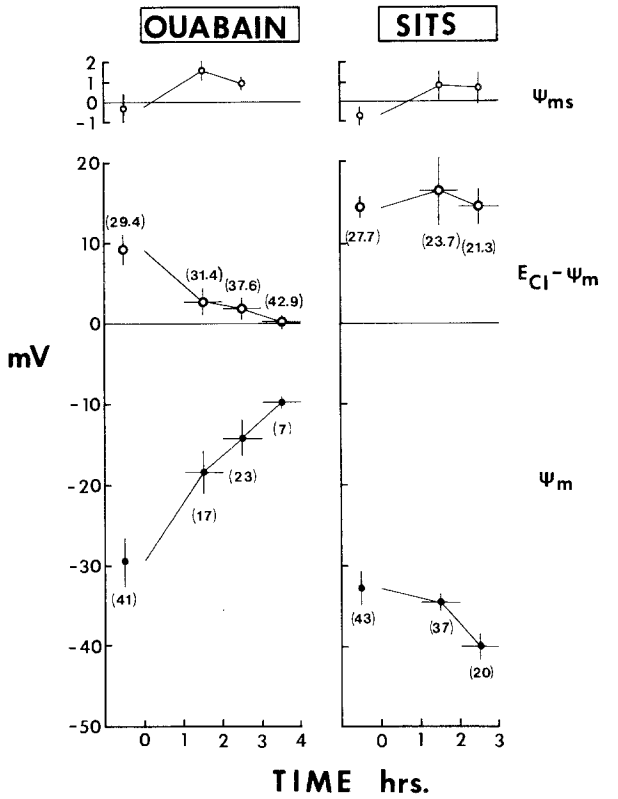

Fig. 9. Effect of 1 mM ouabain (left) and 1 mM SITS (right) on Cl^- transport. The inhibitors were added at $t=0$. Upper traces: transmural PD (ψ_{ms}). Middle trace: Cl^- accumulation potential ($E_{Cl}-\psi_m$) with a^i_{Cl} values in parentheses. Lower trace: mucosal membrane potential (ψ_m) with number of cells impaled in parentheses. Average of values from 5 animals in each series

However, while Cl^- was accumulated in tissues examined in the spring, in most cells of most tissues examined in the winter Cl^- was in electrochemical equilibrium (i.e. $E_{Cl}=\psi_m$). This observation confirms an earlier report that Cl^- is not actively accumulated in animals examined in the winter [22]. The following results pertain to those animals examined in the spring which were actively accumulating Cl^- when bathed in normal medium.

Effect of Serosal Ouabain and Disulfonic Stilbene

In Fig. 9 is shown the effect on Cl^- accumulation and ψ_m of addition to the serosal medium at time $t=0$ of ouabain (left) or the stilbene SITS (right). One to two hours after ouabain addition E_{Cl}-ψ_m was reduced significantly and in 3 to 4 hr accumulation was eliminated. Simultaneously ψ_m was greatly reduced, the largest decline occurring during the period (0 to 2 hr) when $E_{Cl}-\psi_m$ was falling. The serosa negative transepithelial potential (ψ_m) reversed polarity after exposure to ouabain. These results are consistent with the earlier demonstration that ouabain inhibits electrogenic Cl^- absorp-

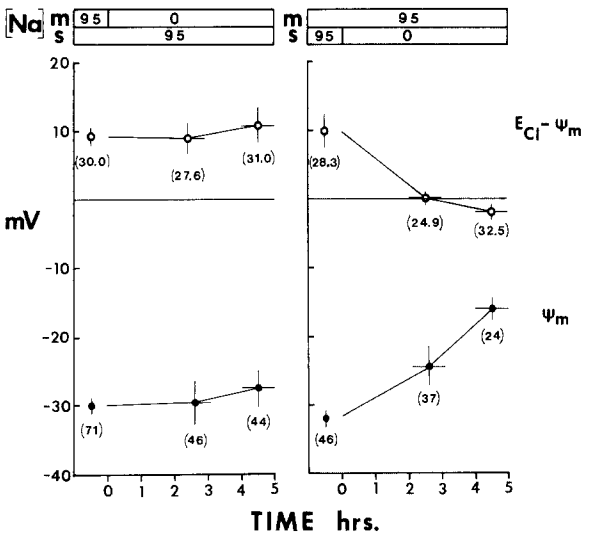

Fig. 10. Effect of unilateral Na replacement on Cl^- transport. Na was replaced in the mucosal (*m*) compartment (left, $n=7$) or serosal (*s*) compartment (right, $n=6$)

tion by *Amphiuma* small intestine [23] and indicate that active Cl^- uptake across the luminal membrane is also blocked.

In common with ouabain, SITS, which also completely blocks net Cl^- absorption [23], also reversed the polarity of ψ_{ms} (Fig. 9, right). In contrast Cl^- accumulation was unaltered even after several hours exposure. Furthermore, the mucosal membrane potential became hyperpolarized. Since net Cl^- absorption is abolished by SITS [23] and the Na^+-stimulated Cl^- flux was blocked by disulfonic stilbenes as well (Table 3), then the continued accumulation of Cl^- in the presence of SITS indicates that the disulfonic stilbenes specifically block a basolateral exit process (specifically Cl^-/HCO_3^- exchange) rather than the active uptake process at the luminal membrane. Furthermore, since a^i_{Cl} was not elevated by SITS in the steady state then the outward flux of Cl^- across the basolateral membrane ($J^{Cl}_{c\to s}$) is not a major factor in determining the steady-state value of a^i_{Cl}. It follows that a^i_{Cl} is greatly influenced by the luminal permeability to Cl^-, i.e. that the flux of Cl^- from cell to mucosal medium ($J^{Cl}_{c\to m}$) exceeds $J^{Cl}_{c\to s}$.

Unilateral Replacement of Medium Na^+

In Fig. 10 it is seen that Cl^- accumulation was unchanged (left) upon replacement of Na^+ with choline in the mucosal perfusate but declined when Na^+ was replaced in the serosal perfusate (right). Two to three hours after serosal Na^+ replacement, Cl^- accumulation was eliminated. The mucosal membrane potential was similarly affected, declining when serosal Na^+ was replaced but remaining unchanged upon replacement of mucosal Na^+. These results are unequivocal in their support of the view that Cl^- accumulation is dependent upon the presence of Na^+, specifically in the serosal perfusate for active accumulation of Cl^-. Furthermore, the parallels between the magnitude of Cl^- accumulation ($E_{Cl}-\psi_m$) and the mucosal membrane potential observed in Figs. 9 and 10 are suggestive that Cl^- is accumulated by an electrogenic process.

Discussion

The present results add two new lines of evidence to that previously reported in support of the view that the Na^+ dependence of intestinal Cl^- absorption in *Amphiuma* is due to a Na^+ requirement at the basolateral rather than the luminal membrane of the intestinal absorptive cell. Previously we showed that the ability of Cl^- added to the luminal fluid to stimulate HCO_3^- entry into the mucosal fluid (i.e. HCO_3^- secretion) required Na^+ in the serosal rather than the mucosal fluid [26]. Thus Na^+ was not necessary at the luminal membrane to enhance Cl^- entry into the cell but instead seemed to be required for the exchange of Cl^- for HCO_3^- at the basolateral membrane. Support for this conclusion was obtained in the present study by two independent approaches. First, it was shown that the absorptive flux of Cl^- was stimulated much more by addition of Na^+ to the serosal fluid than by addition to the mucosal fluid (Table 2). Second, it was shown that active Cl^- accumulation is absolutely dependent on the presence of Na^+ in the serosal but not the mucosal solution (Fig. 10). These results are consistent with the earlier observation that short-circuited *Amphiuma* small intestine does not absorb Na^+ in the absence of transported organic solutes [23]. The results call into question the energetics underlying active accumulation of Cl^- across the luminal membrane and point to a role for Na^+ in basolateral Cl^-/HCO_3^- exchange.

Luminal Cl^- Uptake: Independence from Mucosal Na^+

The labeled Cl^- flux measurements and the Cl^- microelectrode measurements both pointed to a lack of involvement of luminal Na^+ in Cl^- uptake. While $J^{Cl}_{m\to s}$ was stimulated more by serosal Na^+ than by mucosal Na^+ this result alone does not rule out the possibility that Na^+ is required at both membranes for Cl^- uptake into the cell. After

10 mM Na^+ is added to the serosal medium in the chambers employed for the flux measurements the fixed luminal volume gains Na^+ at the rate of 1 μM/min as determined in a previous study under nearly identical conditions [26]. Thus, in 2 hr the luminal [Na] may reach 0.12 meq/liter. This concentration is two orders of magnitude lower than the K_m for the Na dependency of the Cl^- current Fig. 2*b*). Nevertheless this low concentration of luminal Na could be playing a permissive (e.g. cotransport) role in concert with a predominant role of Na^+ at the basolateral membrane. From the ^{36}Cl flux measurements alone this possibility cannot be excluded. However, arguing strongly against this notion Cl^- accumulation was unaltered (i.e. $E_{Cl} > \psi_m$) when mucosal Na^+ was completely replaced with Na^+-free media in a well-perfused chamber that would not permit the development of even small luminal concentrations of Na. In contrast, replacement of serosal Na^+ alone or replacement of mucosal and serosal Na^+, as performed in a previous study [22], eliminated Cl^- accumulation ($E_{Cl} = \psi_m$). Thus active Cl^- accumulation into the cell and net transcellular transport depends on the presence of Na^+ only at the basolateral membrane. Interestingly, the K_m for the dependence of the Cl^--induced I_{sc} on Na^+ (12.4 mM) was very similar to the K_m (10.8 mM) for the dependence of the HCO_3^--secretory flux on Na^+ derived from an earlier study [26]. This suggests that Na^+ interacts through the same site on the serosal membrane to induce HCO_3^- secretion and Cl^- absorption.

Luminal Cl^- Uptake: Possible Primary Active Transport

In the absence of any support for mucosal Na-Cl cotransport alternate sources of energy for Cl^- uptake must be considered. It is possible that Cl^- uptake is linked to the exit of HCO_3^-. The role of the gradient of HCO_3^- at the luminal membrane has not been examined experimentally. A 1-for-1 Cl^-/HCO_3^- exchange would be electrically silent and thus inconsistent with the electrogenic nature of Cl^- absorption. However, it is conceivable that as a consequence of electroneutral exchange a diffusion potential for an ion develops within the mucosa. Alternatively, Cl^- uptake may be electrogenic via either 1) an uncoupled, primary Cl^- pump driven by the energy of ATP [23, 26], or 2) a 2-for-1 Cl^-/HCO_3^- exchange. If Cl^- uptake is rheogenic by either mechanism, then inhibition of uptake should depolarize the brush border membrane. As predicted, the luminal membrane was markedly depolarized under the two conditions which resulted in elimination of active Cl^- accumulation (exposure to serosal Na^+-free media and exposure to ouabain). Simultaneous replacement of Na^+ in both mucosal and serosal fluids was earlier shown to depolarize the luminal membrane [23]. On the other hand Cl^- accumulation was unaltered and ψ_m hyperpolarized when Cl^- transport was blocked by exposure to serosal SITS. Thus, the electrical observations are consistent with the presence of a rheogenic transport of chloride ions into the intestinal absorptive cells. A possible source of metabolic energy for Cl^- uptake is the anion-stimulated ATPase activity reported for rat intestinal brush border membrane [13].

The hyperpolarization of the luminal membrane by SITS may be the result of a reduced outward HCO_3^- current at the luminal membrane. The driving forces promoting HCO_3^- efflux and the mode of transport across the luminal membrane have not been defined. A passive movement of HCO_3^- driven through conductive channels has been proposed [23]. Stilbene inhibition of basolateral Cl^-/HCO_3^- exchange should reduce intracellular HCO_3^- and thereby reduce the chemical driving force for HCO_3^- exit across the luminal membrane. In fact SITS significantly reduces the HCO_3^- secretory flux into the intestinal lumen when measured by titration [14]. From these considerations it is conceivable that luminal hyperpolarization is the consequence of a reduced outward HCO_3^- current. The SITS-induced hyperpolarization may also be due to inhibition of an outward depolarizing anionic current at the basolateral membrane. This would be reflected in ψ_m since ample evidence indicates that luminal and basolateral membranes of low resistance epithelia are electrically coupled.

Serosal Exit Via Cl^-/HCO_3^- Exchange

Evidence that serosal Cl^- exit from the intestinal cell is linked to HCO_3^- entry is considerable. Cl^- absorption is blocked by replacement of medium HCO_3^- [24] and conversely HCO_3^- secretion is reduced by replacing medium Cl^- [14], specifically Cl^- in the luminal medium [26]. Furthermore the disulfonic stilbenes inhibit both HCO_3^--dependent Cl^- absorption [23] and Cl^--dependent HCO_3^- secretion [14]. The latter evidence tends to rule out electrodiffusional coupling between the anions. Of considerable importance the present results provide strong support that the disulfonic stilbenes act directly to inhibit basolateral anion exchange since Cl^- accumulation across the luminal membrane was unimpaired.

The Role of Serosal Na^+ in Cl^- Absorption

Does Na^+ in the serosal medium stimulate Cl^- absorption by promoting, in some way, more Cl^- uptake into the cell or does Na^+ accelerate the basolateral exchange process directly? There is evidence for both effects. In regard to the latter, serosal Na^+ stimulates HCO_3^- secretion even in the absence of medium Cl^- [26], suggesting that basolateral Na^+/H^+ exchange (or Na^+/HCO_3^- transport) occurs wherein the energy inherent in the serosal Na^+ gradient drives an efflux of protons. An ion exchange of this type linked to a parallel Cl^-/HCO_3^- antiport may underlie the requirement for Na^+ in the serosal medium. Recently, the stoichiometry of coupling between Na^+, Cl^-, H^+ and HCO_3^- transport at the basolateral membrane of amphibian renal proximal tubule cells has been examined [12]. Whatever the exact nature of the linkage between these ions, the presence of basolateral ion exchange mechanisms alone would not explain the finding that Cl^- accumulation into the cell across the luminal membrane requires Na^+ in the serosal solution. This ability of serosal Na^+ to enhance Cl^- uptake may stem from the change in the intracellular milieu brought on by Na^+-dependent basolateral ion exchange. Specifically the exit of protons in exchange for Na^+ will influence the cytoplasmic pH (pH_i) and HCO_3^- activity ($a^i_{HCO_3}$). There are several ways $a^i_{HCO_3}$ or pH_i may, in turn, influence apical Cl^- accumulation: i) if there is a requirement for the bicarbonate ion at the inner surface of the apical membrane to have Cl^- uptake, ii) if mucosal HCO_3^- exit is linked to Cl^- entry on a carrier, e.g. 2 Cl^-:1 HCO_3^-, and iii) if intracellular HCO_3^- or pH_i controls a passive Cl^- efflux across the luminal membrane. In regard to the last point we have reported evidence that a back leak of Cl^- across the luminal membrane is accelerated by elevation of intracellular cyclic AMP [24]. Evidence of a Cl^- conductive flux in vesicles from rat brush border membranes has also been reported [15]. Lastly, it is possible that basolateral Na/Ca exchange influences luminal Cl^- transport by regulating cytoplasmic calcium. At present we cannot distinguish between the plethora of models which could account for the interaction between serosal Na^+ and the transport of Cl^- and HCO_3^-.

The net effect of these processes is the electrogenic absorption of Cl^- in exchange for HCO_3^- from the blood. *In vivo* these transport processes would serve to absorb Na^+ passively through paracellular pathways driven by the transepithelial voltage generated by Cl^- transport. In this respect the small intestine and other epithelia which absorb Cl^- electrogenically [2] are in direct contrast with those epithelia (usually high resistance) which apparently absorb Cl^- passively as a consequence of electrogenic Na^+ absorption. The lack of direct coupling between Na^+ and Cl^- allows more independent control of Cl^- transport and linkage to the transport of other ions, especially HCO_3^-.

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