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Coupled Sodium-Chloride Influx across Brush Border of Flounder Intestine

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Summary. Measurements of the unidirectional influxes of Na and Cl from the mucosal solution into the epithelium (J_{me}) of flounder intestine under short-circuit conditions reveal the presence of a coupled NaCl influx process at the brush border membrane which appears to be essential for the absorption of these ions. J_{me}^{Cl} and J_{me}^{Na} were inhibited by replacing Na or Cl, respectively, in the bathing media with non-transported ions which also reduced the short-circuit current (I_{sc}) to near-zero values. Addition of furosemide to the mucosal solution alone inhibited the I_{sc} and reduced J_{me}^{Cl} and J_{me}^{Na} under control conditions, but not in the absence of Na or Cl, respectively. The reductions in J_{me}^{Cl} and J_{me}^{Na} elicited by ion replacement or furosemide were approximately equal, suggesting that the coupled influx mechanism mediates a one-for-one entry of these ions into the cell from the mucosal solution. Furosemide inhibited Cl absorption by reducing the unidirectional Cl flux from mucosa to serosa, consistent with its inhibition of the influx process. As in other epithelia, coupled NaCl influx is inhibited by cyclic AMP, which accounts for the decrease in Cl absorption elicited by cyclic nucleotides. These results support the notion that *transcellular* NaCl transport is a neutral process and that the serosa-negative transepithelial electrical potential difference and preponderance of Cl over Na absorption under short-circuit conditions result from dissimilar permeabilities of the paracellular pathway to Na and Cl.

Studies by Field and collaborators [8] of ion transport by *in vitro* flounder (*Pseudopleuronectes americanus*) intestine suggested an obligatory coupling between the absorptive fluxes of Na and Cl. Support for this notion was derived from ion replacement studies carried out under short-circuit conditions: replacement of Na with choline abolished active Cl absorption, and, conversely, replacement of Cl with SO_4 and mannitol abolished active Na absorption. In the absence of either Na or Cl, the

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spontaneous transepithelial electrical potential difference (ψ_{ms}) was also reduced to zero. Additional evidence of the interaction between Na and Cl transport emerged from the finding that both ψ_{ms} and active Cl absorption are abolished by addition of ouabain to the serosal bathing solution alone.

To reconcile these observations with the fact that, under short-circuit conditions, the rate of Cl absorption is almost 3 times that of Na, Field *et al.* [8] proposed that a process of one-for-one *transcellular* NaCl transport might be obscured by dissimilar permeabilities of the paracellular pathway to these ions. Thus, under short-circuit conditions, much of the Na transported into the lateral intercellular spaces from the cells may recycle into the mucosal solution via Na-selective tight junctions, thereby reducing the rate of *transepithelial* Na transport to a fraction of that for Cl. According to this model, the processes responsible for *transcellular* NaCl transport by flounder intestine may resemble those of rabbit gallbladder [9] where the obligatory coupling between Na and Cl absorption can be attributed to the presence of a neutral NaCl influx process (J_{me}^{NaCl}) at the mucosal membranes which mediates a one-for-one entry of these ions from the mucosal solution into the cells. The results of the present study, employing direct determinations of unidirectional Na and Cl influxes from the mucosal solution into the epithelium of flounder intestine, indicate that a coupled NaCl entry process is responsible for the interaction between transepithelial Na and Cl transport by this tissue. The effects of furosemide and cyclic 3′, 5′-AMP (cAMP) on J_{me}^{NaCl} were also evaluated.

Materials and Methods

Methods for obtaining fish and preparing mucosal sheets of intestine stripped of serosal muscle were those described by Field *et al.* [8]. The Ringer's solution employed for these studies contained (mM): Na, 168; Cl, 75; SO_4, 39; HCO_3, 20; K, 5; $HPO_4 - H_2PO_4$, 2; Ca, 1; Mg, 1; mannitol, 35; and had a pH of 8.0 at 15 °C when gassed with 1% CO_2 – 99% O_2. This half-normal Cl Ringer's was used in an attempt to reduce diffusional Cl fluxes while retaining near-normal rates of Cl absorption [8]. Sodium-free solutions were prepared by substituting choline Cl for NaCl, Tris SO_4 for Na_2SO_4 and choline HCO_3 for $NaHCO_3$. Chloride-free media contained Na_2SO_4 and mannitol as replacements for NaCl.

Transepithelial Cl fluxes were determined under short-circuit conditions as described previously [8]. Bidirectional fluxes were measured during a 30-min control flux period followed by a second 30-min period in the presence of furosemide (10^{-3} M). Twenty minutes were allowed to elapse between control and experimental flux periods to assure the achievement of a new steady state. This overall procedure is justified by prior observations that the bidirectional Cl fluxes remain constant over a 3-hr period [8].

Unidirectional Na and Cl influxes (J_{me}^{Na} and J_{me}^{Cl}) were determined under short-circuit conditions as described by Frizzell and Turnheim [12]. Mucosal strips were mounted horizontally in chambers similar to those described previously [12] except that the serosal (lower) compartment consisted of a gas-lift circulating system which rapidly mixed the serosal solution. During short-circuiting, appropriate correction was made for fluid resistance. Isotopic test media contained either ^{22}Na or ^{36}Cl and ^{3}H-PEG as the extracellular marker. The chamber rested on a thermoelectric device (Cambion, Cambridge, Mass.) whose surface temperature was feedback-controlled from a thermistor probe placed in the serosal solution. In this manner, the serosal solutions were maintained at 15 °C, but the mucosal (upper) solutions were slightly warmer ($\leqq 4$ °C) during the preincubation period. However, the isotopic test solutions were separately maintained at 15 °C so that, during the actual influx measurement, there was little or no difference in temperature across the tissues. The temperatures of both bathing media were routinely monitored using a Yellow Springs Tele-thermometer (Yellow Springs, Ohio).

Tissues were preincubated for 20–30 min in media whose composition was identical to that employed for the subsequent influx determinations. Initial studies were performed to determine the time-course of ^{22}Na and ^{36}Cl uptake from the mucosal solution; the results of these experiments are illustrated in Fig. 1. The Cl uptakes are lower than those of Na at each exposure time, reflecting the concentrations of these ions in the Ringer's solution (168 mM Na, 75 mM Cl). For both Na and Cl, uptake is a linear function of time for at least 1 min. As discussed previously [11, 19], these results strongly suggest that PEG is an adequate marker of the extracellular space and that reliable estimates of J_{me}^{Na} and J_{me}^{Cl} could be obtained from a 45-sec exposure to the test media, which was chosen as the test period for subsequent studies.

Furosemide was a generous gift of Hoechst-Roussel Pharmaceuticals (Somerville, N.J.); dibutyryl cyclic AMP (DBC) and theophylline were obtained from Sigma. All radioisotopes were purchased from New England Nuclear. Other chemicals were of reagent grade. Tris SO_4 was prepared by titrating Tris base with H_2SO_4.

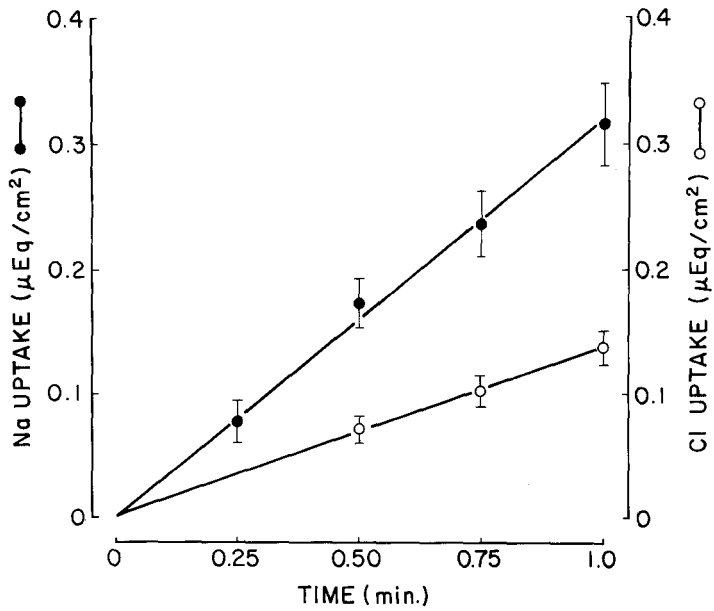

Fig. 1. Time courses of Na and Cl uptake from the mucosal solution. Each point is the mean ± SEM of 6 determinations

All results are expressed as the mean ± SEM. Statistical comparisons were made by using the Student t test; a value of $p < 0.05$ was considered significant.

Results

Effects of Furosemide on Short-Circuit Current (I_{sc}) and Transepithelial Cl Fluxes

Furosemide rapidly inhibited ψ_{ms} and the I_{sc} across flounder intestine when added to the mucosal solution alone. The dose-response relation between I_{sc} and the mucosal solution furosemide concentration is illustrated in Fig. 2. The concentration of furosemide that produced a 50% inhibition of I_{sc} was in the range of 10^{-6}–10^{-5} M; approximately 90% inhibition was observed at 10^{-3} M. It is unclear at the present time whether a small component of I_{sc} is furosemide-insensitive or whether a concentration of 10^{-3} M is submaximal; the effects of higher concentrations were not explored. The inhibition of I_{sc} produced by addition of the diuretic to the mucosal solution was complete within 1 min, suggesting a direct interaction with the process(es) responsible for Na and/or Cl transport across the mucosal membrane.

Bidirectional Cl fluxes were determined under short-circuit conditions in the presence and absence of 10^{-3} M furosemide added to the mucosal solution alone; the results of these studies are given in Table 1. Again, the inhibition of I_{sc} elicited by this concentration of furosemide

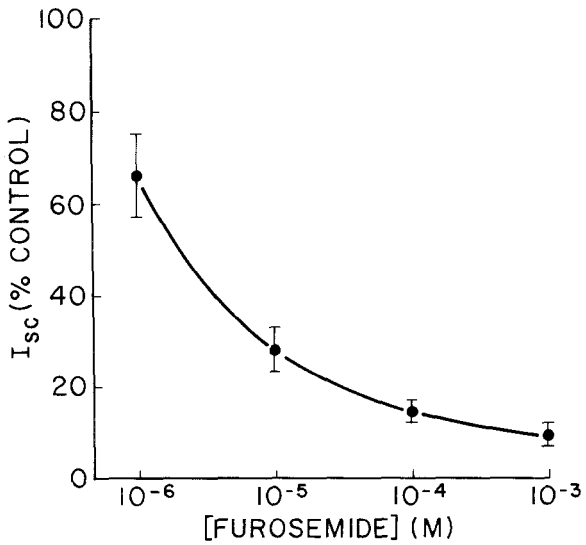

Fig. 2. Short-circuit current as a function of mucosal solution furosemide concentration. Each point represents the mean ± SEM of 7 determinations

Table 1. Effect of furosemide on unidirectional Cl fluxes across flounder intestine

	J_{ms}^{Cl}	J_{sm}^{Cl}	J_{net}^{Cl}	I_{sc}	G_t
Control	5.8±4	1.8±0.3	4.0±0.3	−2.4±0.4	24±1
+Furosemide	3.2±0.2[a]	1.7±0.3	1.5±0.3[a]	−0.6±0.1[a]	23±1

All values are expressed in μeq/cm² hr except G_t in mmhos/cm². J_{ms}^{Cl} represents the unidirectional flux of Cl from mucosa-to-serosa, J_{sm}^{Cl} designates that from serosa to mucosa, and J_{net}^{Cl} is the difference between these oppositely directed fluxes. Furosemide was added as a stock solution to the mucosal compartment alone; final concentration, 10^{-3} M. Values are from 7 experiments on paired tissues.

[a] Different from control value; $p<0.05$.

was incomplete. Chloride absorption was reduced by furosemide, and the decline in J_{net}^{Cl} could be entirely attributed to a decrease in the unidirectional Cl flux from mucosa (m) to serosa (s). Furosemide had no effect on tissue conductance (G_t).

Unidirectional Na and Cl Influxes

The effects of furosemide and Na-free media on J_{me}^{Cl}, I_{sc} and G_t are presented in Table 2. Both I_{sc} and J_{me}^{Cl} were inhibited by the diuretic. Thus, the furosemide-induced reduction in J_{ms}^{Cl} noted in Table 1 can be attributed to inhibition of Cl entry from the mucosal solution into the cells across the brush border membrane. Removal of Na from the bathing media elicited reductions in J_{me}^{Cl} and I_{sc} that were not significantly different from those produced by furosemide. Finally, in tissues exposed to Na-free media, furosemide had no effect on J_{me}^{Cl}.

Table 2. Effect of furosemide and Na-free media on chloride influx

	J_{me}^{Cl}	I_{sc}	G_t
Control	8.1±0.5	−4.0±0.5	24±1
+1 mM furosemide	4.3±0.5[a]	−0.6±0.2[a]	25±1
Na-free	5.2±0.5[a]	−0.3±0.1[a]	6±1[a]
Na-free +1 mM furosemide	4.3±0.8[a]	−0.4±0.1[a]	9±1[a]

All values are expressed in μeq/cm² hr except G_t in mmhos/cm². Tissues were exposed to furosemide by replacing the preincubation solution with one of identical electrolyte composition but also containing 10^{-3} M furosemide 5 min prior to the influx determination. The test media also contained furosemide. Each value represents the mean ±SEM of 8 determinations.

[a] Different from control value; $p<0.05$.

Table 3. Effect of furosemide and Cl-free media on sodium influx

	J_{me}^{Na}	I_{sc}	G_t
Control [14]	22±1	−3.1±0.5	26±2
+1 mM furosemide [10]	17±1[a]	−0.5±0.1[a]	27±2
Cl-free [12]	15±1[a]	0.5±0.2[a]	20±2
Cl-free +1 mM furosemide [8]	16±2[a]	0.4±0.1[a]	24+3

See legend to Table 2. The number of observations is given in parentheses.
[a] Different from control value; $p<0.05$.

The effects of furosemide and Cl-free media on Na influx are presented in Table 3. Exposure to furosemide or Cl-free solutions decreased J_{me}^{Na}; their inhibitory effects were similar in magnitude. In addition, furosemide had no effect on Na influx in the absence of Cl. The presence of a small positive I_{sc} in Cl-free media (reflecting a serosa-positive ψ_{ms}) most likely represents the presence of diffusion potentials which had not been entirely dissipated at the time these measurements were made (ca. 20–30 min following exposure to Cl-free solutions). These have been noted in prior studies [8] and will decay to zero if given sufficient time.

The results presented in Tables 2 and 3 strongly suggest that 4–5 μeq/cm² hr of the influxes of Na and Cl are coupled in an obligatory fashion and that the inhibitory effect of furosemide is exerted upon this coupled entry process. The effects of furosemide cannot be attributed solely to a change in the electrical potential difference across the mucosal membrane since this would have opposing influences on J_{me}^{Na} and J_{me}^{Cl}; nor can its effects be readily attributed to changes in passive Na or Cl influxes since furosemide had no effect on tissue conductance.

Effect of cAMP

The results of previous studies indicate that cAMP inhibits active Cl absorption by flounder intestine[1]. The effects of dibutyryl cAMP (DBC) and theophylline on J_{me}^{Cl} were evaluated (Table 4). The experiments were performed in the presence and absence of furosemide for reasons that will be discussed below. As shown in Table 4, DBC and theophylline

[1] Field, M., Smith, P.L., Bolton, J.E. 1978. Ion transport across the isolated intestinal mucosa of the winter flounder, *Pseudopleuronectes americanus:* Effects of cyclic AMP. (*unpublished*).

Table 4. Effect of furosemide and DBC plus theophylline on chloride influx

	J_{me}^{Cl}	I_{sc}	G_t
Control	8.9±0.4	−4.2±0.7	27±2
+1 mM Furosemide	4.8±0.3[a]	−0.3±0.1[a]	25±1
+0.25 mM DBC +5 mM Theophylline	7.5±0.5	−0.3±0.3[a]	28±1
+1 mM Furosemide +0.25 mM DBC +5 mM Theophylline	6.0±0.6[a]	0.2±0.1[a]	29±2

See legend, Table 2. Each value represents the mean ± SEM of 8 determinations.
[a] Different from control value; $p<0.05$.

reduced the I_{sc} as previously reported by Field et al.[2]. In addition, J_{me}^{Cl} tended to decrease, but fell short of reaching statistical significance ($0.5<p<0.1$). However, the inhibitory effect of furosemide was significantly smaller in the presence of DBC and theophylline ($p<0.05$). The inhibition of J_{me}^{Cl} elicited by furosemide under control conditions was 4.1 μeq/cm² hr and in good agreement with the results presented in Table 2; however, in the presence of DBC and theophylline, its inhibitory effect was only 1.5 μeq/cm² hr. Therefore, the furosemide-sensitive component of J_{me}^{Cl} is reduced by DBC and theophylline.

Discussion

Coupled NaCl Influx

The results of this study indicate that the absorption of Na and Cl by flounder intestine can be attributed to the operation of a coupled NaCl influx process at the mucosal membrane which is inhibited by furosemide. When the bathing media are rendered Na-free, Cl entry from the mucosal solution into the epithelium is reduced (Table 2), and net Cl absorption is abolished [8]. Conversely, exposure to Cl-free bathing solutions results in a reduction in Na influx from the mucosal solution into the tissue (Table 3) and also abolishes Na absorption [8]. In addition, the action of furosemide appears to be restricted to the coupled NaCl entry mechanism inasmuch as its inhibitory effects on Na and Cl influxes require the presence of both ions in the bathing media. In parallel with its inhibition of J_{me}^{NaCl}, furosemide reduces Cl absorption, solely as a consequence of inhibiting the unidirectional Cl flux from

[2] *Ibid.*

mucosa to serosa (Table 1). Thus, the effects of agents or conditions which influence NaCl influx are directly correlated with their effects on transepithelial electrolyte transport, and this suggests that the coupled NaCl entry process at the brush border plays an essential role in the absorption of Na and Cl by this tissue. Recently, Eveloff, Field, Kinne and Murer (*personal communication*) demonstrated that Na uptake by isolated brush border vesicles obtained from intestinal mucosa of winter flounder was decreased by furosemide or Cl-free media. These effects could not be attributed to changes in the electrical potential difference across the vesicular membrane, implying that Cl-dependent Na uptake is electrically neutral. It is of interest that Smith *et al.* [20] were unable to detect a Na-dependent component of Cl influx using intestine of the European flounder, *Platichthys flesus*. This discrepancy could arise from the difference in species or experimental techniques employed. Inasmuch as the chamber used by Smith *et al.* [20] had no serosal compartment, the composition of the fluid in contact with the serosal surface of the tissue is unknown, as is ψ_{ms}. Therefore, it is possible that alterations in ψ_{ms} may complicate the interpretation of their ion replacement studies, due to changes in the passive component of Cl influx.

The interaction between Na and Cl influxes observed in the present studies appears to involve a one-for-one uptake of Na and Cl into the cells from the mucosal solution since the inhibitions of J_{me}^{Cl} and J_{me}^{Na} elicited by Na- or Cl-free media or by furosemide are approximately equal. Thus, the process responsible for NaCl transport across the brush border of flounder intestine resembles those of rabbit gallbladder [9] and ileum [15], tissues in which direct evidence of a neutral, coupled NaCl influx mechanism at the mucosal membrane has been presented. Indeed, neutral NaCl transport processes have been suggested for absorptive epithelia of a number of species ranging from arthropods through mammals [1, 13], and furosemide-induced inhibition of coupled NaCl absorption by *in vivo* rat ileum was recently described by Humphreys [14].

In rabbit gallbladder, the energy for active Cl absorption appears to be derived from its interaction with Na and the energy inherent in the Na gradient across the mucosal membrane. Support for this view was initially derived from the finding that exchangeable cell Cl concentration declined toward the value expected for an equilibrium distribution of Cl between the cells and the external bathing solutions [9]. More recently, Duffey *et al.* [5] employed Cl-selective, liquid ion exchanger, micro-electrodes in determining intracellular Cl activities, $(Cl)_c$, of rabbit gall-

bladder epithelial cells. Under transporting conditions, $(Cl)_c$ was 2.3 times the value predicted for an equilibrium distribution. When the tissue was bathed in Na-free Ringer's, $(Cl)_c$ rapidly declined to the value expected for an equilibrium distribution of Cl between the cells and the bathing media. The electrochemical potential difference for Na across the mucosal membrane appears to be more than sufficient to energize cellular Cl accumulation by the epithelial cells of rabbit gallbladder [5]. This Na gradient, in turn, is maintained by an energy-dependent, ouabain-sensitive active Na extrusion mechanism at the basolateral membrane [9]. Net chloride exit from the cell across the basolateral membrane is in the direction of a favorable electrochemical potential difference for this ion and, therefore, no *direct* link between Cl transport and metabolic energy conversion need be invoked to account for active Cl absorption. Spring and Kimura [22] have reached similar conclusions with regard to the mechanism of Cl absorption by *Necturus* proximal tubule. Moreover, Cl activities of flounder intestinal cells have recently been determined using the techniques previously applied to rabbit gallbladder (M.E. Duffey, R.A. Frizzell, S.M. Thompson, and S.G. Schultz, *unpublished*). The ratio of observed cell Cl activity to that predicted for equilibrium averaged 3.4 in the presence of Na but fell to 1.3 after Na was removed from the bathing media. These findings, together with those of the present study, strongly suggest that cellular Cl accumulation and transepithelial Cl transport by flounder intestine are energized by the Na gradient across the mucosal membrane.

Effect of Cyclic AMP

In other epithelia where coupled NaCl influx mechanisms have been identified, cyclic AMP reduces Cl absorption by inhibiting the coupled NaCl entry process [9, 15]. This is reflected by a decline in the unidirectional Cl flux from mucosa to serosa (J_{ms}^{Cl}) [6, 16]. In flounder intestine, cyclic AMP also inhibits Cl absorption, but J_{ms}^{Cl} is not significantly reduced; rather, the unidirectional flux of Cl from serosa to mucosa (J_{sm}^{Cl}) is enhanced[3]. This increment in J_{sm}^{Cl} appears to reflect a cyclic AMP-mediated increase in transepithelial Cl permeability[4] which tends to obscure any effect of this agent on J_{ms}^{Cl}. Since alterations in Cl permeability would also complicate an analysis of the effect of cyclic

[3] *Ibid.*

[4] *Ibid.*

AMP on coupled NaCl influx, these studies were performed in conjunction with furosemide, which appears to act as a selective inhibitor of the coupled entry process. If the furosemide-sensitive component of Cl influx represents J_{me}^{NaCl}, then it is clear from the data in Table 4 that the coupled entry mechanism is inhibited by DBC and theophylline, in agreement with results obtained from other tissues [7, 13]. The inhibition of J_{me}^{NaCl} was not complete, but at comparable levels of cyclic AMP plus theophylline, transepithelial Cl transport is also not reduced to zero[5]. It is possible that the use of higher concentrations might produce a greater degree of inhibition, as was observed for rabbit gallbladder [9].

Nevertheless, these findings, together with those of Field *et al.*[6], suggest that cyclic AMP does inhibit coupled NaCl entry across the brush border of flounder intestine, but that the anticipated decrease in J_{ms}^{Cl} is offset by an increase in Cl permeability. It is of interest that neither rabbit gallbladder nor flounder intestine display the capacity for active Cl secretion that characterizes the response of mammalian small [6] and large [10] intestine to the cyclic nucleotide. As suggested previously [7, 13], this may be related to the absence of crypt regions in the former tissues.

Relation of Coupled NaCl Influx to NaCl Absorption

A coupled NaCl influx process which appears to bring about a one-for-one entry of Na and Cl across the brush-border membrane plays an essential role in absorption of these ions by flounder intestine. Yet, under short-circuit conditions, the rate of transepithelial Cl transport exceeds that of Na by a factor of nearly three. These findings lend further support to the notion of Field *et al.* [8] that a dissociation of neutral transcellular NaCl transport occurs at the level of the paracellular pathway because of inhomogeneities in its permselective properties. The discrepancy between Cl-dependent Na entry (Table 3) and net Na transport [8] indicates that a "recycling" of "transported" Na into the mucosal solution occurs. It seems highly unlikely that the cellular pathway is involved in this backflux of Na since the electrochemical potential difference for Na across the mucosal membrane undoubtedly favors Na entry and appears to be large: Recent measurements of the electrical potential difference across the brush-border membrane of flounder intestine yielded a value

[5] *Ibid.*

[6] *Ibid.*

of 69 ± 2 mV ($n = 15$), cell interior negative with respect to the mucosal solution (M.E. Duffey, R.A. Frizzell, S.M. Thompson and S.G. Schultz, *unpublished*). Thus, it is more likely that the junctional region at the apical boundary of the lateral intercellular space permits a backflux of transported Na into the luminal solution, leading to the preponderance of transepithelial Cl over Na transport that is observed under short-circuit conditions [8 and footnote 1].

The ion replacement studies presented in Table 2 may provide some insight into an essential element of this proposal, namely the perm-selective properties of the junctional region. Paracellular pathways provide the major conductive route for passive ion flow across "leaky" epithelia [4, 11], and the junctional regions (*zona occudens*) appear to form the major resistive element of these pathways [4, 21]. Thus, changes in tissue conductance resulting from replacement of Na with an (presumably) impermeable substitute cation should primarily reflect the partial conductance of the junctional pathway to Na. Tissue conductance decreased 75% when Na in the bathing solutions was replaced with choline (Table 3), suggesting that this pathway is highly permeable to Na.

Finally, it is of interest that many of the characteristics of flounder intestine parallel those of the thick ascending limb of the loop of Henle (TALH). This nephron segment displays a lumen-positive transepithelial electrical potential difference and actively absorbs Cl from lumen to peritubular solution [2, 17]. Although Na absorption by the TALH is in the direction of a favorable transtubular electrical potential difference, studies aimed at clarifying the mechanism of Na absorption have been inconclusive [2, 17]. The ratio of bidirectional Na fluxes across the TALH deviated from that predicted by the Ussing flux-ratio equation suggesting a modest rate of active Na absorption. Such studies are complicated by the fact that transepithelial Na permeability is high and markedly exceeds that to Cl, as is the case for flounder intestine. In addition, active Cl transport by the TALH is inhibited by addition of ouabain to the solution bathing the peritubular (serosal) side [2, 17] or by furosemide added to the solution perfusing the lumen [3]. The presence of ouabain-sensitive active Cl absorption and high levels of Na, K-ATPase in this nephron segment [18] are particularly interesting with regard to the model discussed above for Cl absorption by flounder intestine. The possibility of neutral transcellular NaCl transport modified by the paracellular pathway seems worthy of further investigation in the TALH in view of the similar physiologic and pharmacologic properties of these epithelia.

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