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KCl Transport across an Insect Epithelium: I. Tracer Fluxes and the Effects of Ion Substitutions

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Summary. Models for active Cl transport across epithelia are often assumed to be universal although they are based on detailed studies of a relatively small number of epithelia from vertebrate animals. Epithelial Cl transport is also important in many invertebrates, but little is known regarding its cellular mechanisms. We used short-circuit current, tracer fluxes and ion substitutions to investigate the basic properties of Cl absorption by locust hindgut, an epithelium which is ideally suited for transport studies. Serosal addition of 1 mM adenosine 3':5'-cyclic monophosphate (cAMP), a known stimulant of Cl transport in this tissue, increased short-circuit current (I_{sc}) and net reabsorptive ^{36}Cl flux (J_{net}^{Cl}) by 1000%. Cl absorption did not exhibit an exchange diffusion component and was highly selective over all anions tested except Br. Several predictions of Na- and HCO_3-coupled models for Cl transport were tested: Cl-dependent I_{sc} was not affected by sodium removal (<0.05 mM) during the first 75 min. Also, a large stimulation of J_{net}^{Cl} was elicited by cAMP when recta were bathed for 6 hr in nominally Na-free saline (<0.001 to 0.2 mM) and there was no correlation between Cl transport rate and the presence of micromolar quantities of Na contamination. Increased unidirectional influx of ^{36}Cl into rectal tissue during cAMP-stimulation was not accompanied by a comparable uptake of ^{22}Na. J_{net}^{Cl} was independent of exogenous CO_2 and HCO_3, but was strongly dependent on the presence of K. These results suggest that the major fraction of Cl transport across this insect epithelium occurs by an unusual K-dependent mechanism that does not directly require Na or HCO_3.

Key Words insect epithelium · cAMP-stimulated Cl^- transport · locust rectum · K^+-dependent Cl absorption

Introduction

Studies of vertebrate epithelia have demonstrated two mechanisms of secondary active Cl transport: 1) sodium-dependent systems in which Cl enters the cell by NaCl cotransport [6, 7, 27], indirectly coupled via parallel Na/H and Cl/OH exchanges [20, 33] or Na, K, Cl cotransport [10, 21] and 2) bicarbonate-coupled systems in which Cl enters or exits by electroneutral exchange for HCO_3 [19]. Active Cl transport is widespread in invertebrate epithelia and has been identified in several organs of 17 different insect species; however, its properties and underlying mechanisms have not been studied in detail.

Many functions of hindgut epithelia in insects parallel those of the vertebrate kidney and gastrointestinal tract (e.g. absorption of salts, amino acids and water). In locusts, active Cl absorption across the rectum has been demonstrated *in situ* [23] and *in vitro* [34]. This process is normally regulated in the intact locust in response to salt loading or depletion [24] and is stimulated *in vitro* by cAMP or neuroendocrine gland homogenates [29–31], hemolymph from recently fed locusts [11, 24, 32] and the purified neuropeptide "Chloride Transport Stimulating Hormone" (CTSH; [25, 26]). Transport rates are comparable *in vivo* and *in vitro* after stimulation by CTSH or cAMP in Ussing-type chambers containing saline with 200 mM Cl. Net Cl flux *in vitro* is similar to that observed *in situ* when KCl is injected into the ligated rectum. Moreover, the spontaneous transepithelial potential during cAMP stimulation *in vitro* is +30 mV (mucosa positive), similar to that measured *in situ*.

The rectal epithelium of the desert locust *Schistocerca gregaria* is particularly well-suited as a model for detailed studies of ion transport because it has a high rate of salt absorption, large surface area compared to most insect epithelia (60 mm^2), and is robust for *in vitro* studies. A chitinous cuticle covers the mucosal surface and acts as a natural support grid. The transporting columnar cells are large (15 × 100 μm), accept microelectrode impalement readily, and make up the great bulk of the epithelium.

Na- and HCO_3-coupled entry models for Cl up-

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take offer testable predictions: 1) removal of external Na should inhibit Cl entry and transepithelial Cl transport and there should be a positive correlation between the amount of Na contamination in nominally Na-free saline and the amount of "residual" Cl absorption; 2) alterations in the rate of Cl influx across the apical membrane should be paralleled by changes in the rate of Na influx; 3) serosal addition of ouabain should reduce Cl transport indirectly through elimination of the favorable Na electrochemical gradient across the apical membrane; 4) perfusion with CO_2, HCO_3-free saline should inhibit Cl/HCO_3 exchange entry unless HCO_3 is generated from metabolic CO_2 at a sufficiently high rate to supply the exchanger; 5) HCO_3 should appear in the mucosal saline at the same rate and with the same time-course as J_{net}^{Cl}.

In this paper we use tracer fluxes, short-circuit current and ion substitutions and inhibitors to test the predictions of common Na- and HCO_3-coupled models. The results suggest that the major fraction of Cl transport across this epithelium is K-dependent and does not occur by a Na- or HCO_3-coupled mechanism. Some of these findings have appeared in preliminary form [13–15]. The companion paper describes microelectrode studies of the locust rectum, including evidence that there is not a favorable Na gradient to drive Cl entry under some experimental conditions.

Materials and Methods

Animals

Desert locusts, *Schistocerca gregaria* Forskal, were reared in gregarious phase at 50% relative humidity under a 15:9 hr light:dark cycle. Colony temperature oscillated between 40°C (light) and 26°C (dark). Locusts were fed fresh lettuce daily and a dry mixture of alfalfa, bran, yeast and powdered milk. Adult female locusts 18 to 30 days beyond final moult were used because of their larger size.

Flux Chambers

Fluxes were measured in Ussing-type chambers designed to minimize edge damage [34, 35]. The tissue was mounted as a flat sheet over a collar-shaped opening and fastened on the outside of the collar by fine tungsten pins near the base. To form a seal, a rubber "O"-ring was placed around the tissue so that it fit snugly into a shallow groove on the collar above the pins. The area of the opening was 0.196 cm^2. This collar design ensured that no pressure was exerted on the tissue when the two half-chambers were clamped together.

Solutions

Five ml of saline were circulated vigorously in both half-chambers by a gas-lift pump. Salines containing 10 mM HCO_3 were bubbled with 95% O_2: 5% CO_2. These remained at pH 6.8 to 7.0 during experiments. When HCO_3-free saline was stirred with 100% O_2, pH increased on the mucosal side from 7.0 to 7.4. This change was ignored since I_{sc} is not affected by pH over the range 6.2 to 8.0 [12]. Experiments were performed at 22 ± 1°C, a value which is experienced by locusts in the wild.

The composition of salines was based on analyses of locust hemolymph and Malpighian tubule fluid [12] (*see also* [4]). The ionic composition of physiological fluids and experimental salines are given in Table 1. All salines used in this study also contained (mM): alanine (2.9), arginine (1.0), asparagine (1.3), glutamine (5.0), glycine (11.4), histidine (1.4), lysine (1.4), proline (13.1), serine (1.5), tyrosine (1.9), valine (1.8). Glucose (10 mM) was also included. Sodium was replaced with choline in Na-free saline, except where indicated otherwise. All salts and the distilled water were tested for Na contamination using an atomic absorption spectrophotometer. K- and HCO_3-free salines were prepared by omitting the normally small amounts of these ions and adjusting sulfate salts and sucrose to maintain constant Na, Mg, Ca, Cl and osmotic concentration.

A large anion deficit was observed in Malpighian tubule fluid, perhaps the result of dissolved urate or polyanions. Since the anion was not identified, K-methylsulfate was added to prepare "high-K" saline. Methylsulfate had no deleterious effects on I_{sc} when added to normal saline in preliminary experiments. Osmotic concentration of salines was routinely checked using an osmometer (Wide Range, Advanced Instr. Inc., Newton Highlands, Mass.) and for 15 different bulk preparations was 444.2 ± 9.2 mOsm/liter.

Prior to tracer flux measurements, tissues were exposed to Na-, K- or HCO_3-free salines for 4 hr and rinsed at least twice with fresh saline except during Na-free experiments when saline was replaced three times. After turning off voltage clamp, solutions were drained by suction and replaced using a syringe. No transients or artifacts resulted from this method of changing solutions.

To determine trace levels of contamination in Na- and K-free salines during flux studies, an atomic absorption spectrophotometer (AA 120, Varian Techtron, PTY, Ltd. Melbourne, Aust.) was used to analyze for these cations at the beginning and at the end of experiments. Maximal stimulation of rectal transport (1 mM cAMP, final conc.) was obtained by adding 50 µl of a 101 mM cAMP solution to the serosal half-chamber. The Na salt of cAMP was normally used instead of the acid, except in Na-free saline, when a stock solution of cAMP was titrated to neutrality with KOH. Amino acids and cAMP were obtained from Sigma (St. Louis, Mo.).

Electrical Methods

Transepithelial potential (V_t) was measured using a high input impedance differential amplifier (10^{12} Ω; 4253 Teledyne Philbrick, Dedham, Mass.) connected to the chambers via calomel electrodes and 3 M KCl agar bridges. Operational amplifiers (725, National Semi-conductor Corp., Santa Clara, Calif., and 308, Fairchild Inc., Mountain View, Calif.) were used for voltage clamping and to measure I_{sc}, respectively. Both I_{sc} and open-

Table 1. Composition of physiological fluids and experimental salines used to study ion transport across locust rectum

	Concentration (mean ± SE, *n*) (meq liter^{-1})						
	Cl	K	Na	Mg	Ca	HCO_3	pH
Body fluids:							
Hemolymph	106.7 ±14.7(6)	12.2 ±1.6(8)	103.0 ±7.4(9)	24.4 ±5.0(5)	18.4 ±5.2(5)	13.0 ±1.0(6)	7.1 ±0.04(9)
Malpighian tubule fluid	87.6 ±14.3(11)	165.1 ±15.1(11)	46.9 ±7.9(13)	39.3 ±8.4(10)	13.1 ±2.4(11)	—	—
Salines:[a]							
Normal saline	110–114	10	110–114	20	10	10	7.1
"High K" saline	50	140[b]	50	20	10	10	7.1

[a] *See* text for organic constituents.
[b] Added as methylsulfate.

circuit V_t were recorded on a strip chart recorder (220, Soltec Corp., Sun Valley, Calif.). Corrections were made for asymmetries between voltage-sensing electrodes and for resistance of the bathing saline using a circuit adapted from Rothe et al. [28] (*see* ref. [12] for details).

Transepithelial Tracer Fluxes

The protocol for measuring transepithelial tracer fluxes was as follows: Aliquots of stock isotope (10 to 50 μl) were added to one half-chamber (referred to as the hot side). ^{36}Cl (New England Nuclear, carrier-free, 5.9 mCi/g Cl) was added as $H^{36}Cl$ to control and Na-free salines, and as $Na^{36}Cl$ to HCO_3-free saline. These amounts were too small to cause significant changes in [Cl] (<3% error) or pH. One-ml samples were taken from the "cold side" at 15- or 20-min intervals and replaced with cold saline. Radioactivity of the hot side did not change measurably during flux experiments. The tracer activity of the cold side never exceeded 0.5% of that on the hot side, hence no correction was necessary for tracer backflux. Appropriate corrections were made for quenching and for dilution during sampling. Initial flux values, which included equilibration with the tissue pool, were not used in calculations. No attempt was made to measure both unidirectional fluxes on each tissue using ^{36}Cl and ^{77}Br, since selectivity experiments showed that Br is transported at less than ½ the rate of Cl. Nevertheless, differences in I_{sc}, V_t and transepithelial resistance (R_t) were not statistically significant between tissues used for forward and back fluxes; and any small differences are specified in the Results section. To assess permeability due to edge damage, unidirectional fluxes of $^{35}SO_4$ were measured across recta in "high-SO_4" saline (207 meq/liter; *see* SO_4 saline #1, [31]) under control conditions and after sequential additions of 1 mM cAMP and 114 mM NaCl. Forward and back fluxes of $^{35}SO_4$ were not significantly different ($P > 0.2$) suggesting that little if any active SO_4 transport occurs [12]. If we assume that all of the observed $^{35}SO_4$ flux occurs through nonselective edge damage, then we may calculate the upper limit of SO_4 permeability through this pathway to be 3.7×10^{-7} cm sec^{-1}. An upper limit for ^{36}Cl flux due to edge damage (corrected for the relative free-solution mobilities of Cl and SO_4) is <12% of the total Cl backflux and is negligible (1.5%) compared to the rate of net Cl absorption after cAMP stimulation. We conclude that fluxes measured using this chamber design are not significantly influenced by edge damage and independent electrophysiological data in the following paper support this view.

Tracer Influxes across the Apical Cell Border

Rectal sacs were cannulated with polyethylene tubing (PE 90, Parsippany, Dickinson and Co., N.J.), everted, and filled with normal saline as previously described [9]. This method allows measurement of V_t and unidirectional fluxes of ^{22}Na or ^{36}Cl from the mucosal side into epithelial tissue under open-circuit conditions. The following protocol was used: Everted rectal sacs (mucosal side out) were incubated with control saline on both sides for 3 hr. Some sacs were injected with 10 mM cAMP for the final hour. V_t was measured using a high input impedance electrometer (602, Keithley Instr., Cleveland, Ohio) connected by calomel electrodes and 3 M KCl agar bridges.

A layer of porous cuticle covers the mucosal surface of the epithelium, trapping an unstirred layer external to the apical membrane. In order to equilibrate tracers with this dead space, tissues were placed in control saline containing ^3H-mannitol for 20 min, then transferred to saline containing ^3H-mannitol and ^{36}Cl or ^{22}Na for an additional 30 to 45 min at pH 4.5. This pH, which represents the lower limit of the normal physiological range (pH 4.5 to 7.2), rapidly and reversibly inhibits Cl transport in locust rectum *in vitro* [14]. After a timed exposure to the test solution at pH 7.0, tissues were dissected from the cannula so that the subintimal space was open, and recta were blotted on bibulous paper, weighed, macerated in vials containing 1 N KOH, and digested at 80°C overnight. After neutralizing with H_2SO_4, the vials were counted for ^3H-mannitol and ^{36}Cl as previously described. ^{22}Na was counted using an automatic gamma counter (model 4253, Searle). Tritium counts were corrected for activity due to other tracers.

^3H-mannitol was used to estimate extracellular tissue space that was in continuity with the mucosal side during tracer influx measurements. We found that mannitol is not metabolized by

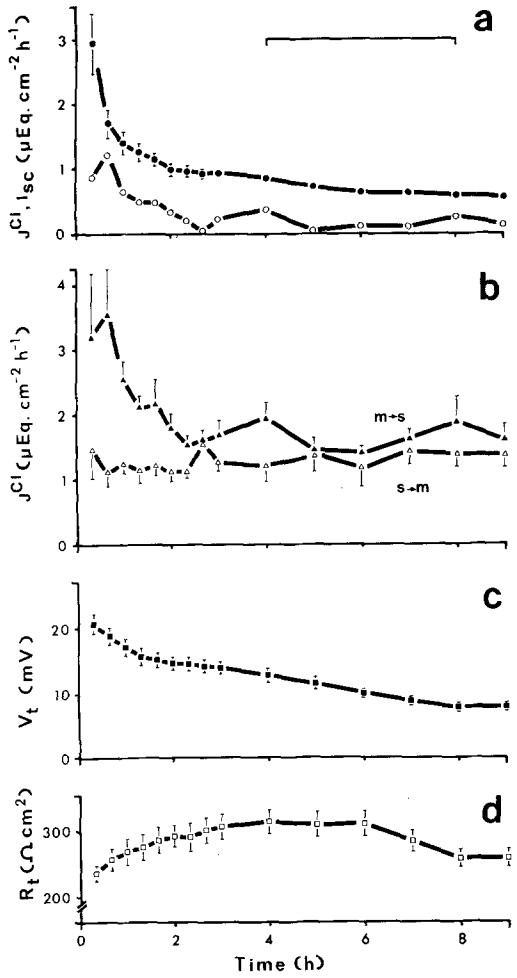

Fig. 1. The approach of isolated, unstimulated rectal tissue to steady-state conditions. (*a*) short-circuit current (●, I_{sc}) and mean net Cl flux (○, J^{Cl}_{net}); (*b*) unidirectional Cl fluxes measured from mucosa to serosa ▲, and from serosa to mucosa △ under I_{sc} conditions; (*c*) spontaneous transepithelial potential (V_t); (*d*) transepithelial resistance (R). All subsequent experiments were performed during the time interval shown by the horizontal bracket as in (*a*). Recta were bathed in normal saline (Table 1) and were left short-circuited except when spontaneous V_t was measured. Means ± SE; n = 6 (J^{Cl}_{ms}, J^{Cl}_{sm}); n = 12 (I_{sc}, V_t, R_t)

locust rectum.[1] Low counts were observed in sac absorbate after 30-min exposure to pH 4.5; however, ratios of $^{36}Cl:^{3}H$ and $^{22}Na:^{3}H$ were similar to those outside the sac, indicating that i) a nonselective shunt was probably responsible for the ^{3}H influx, and ii) ^{3}H-mannitol still provides a reasonable estimate of extracellular ^{36}Cl and ^{22}Na. In control experiments, ^{22}Na and ^{36}Cl did not accumulate measurably inside rectal sacs during 1 to 10-min exposures to tracers at pH 7.0. Finally, cAMP stimulates the influx of ^{36}Cl into rectal tissue sevenfold, providing further evidence that tracer influx into whole tissue is a valid measure of entry into the epithelial cells (Fig. 5).

[1] Six tissues were incubated separately in stoppered test tubes with 1 ml of control saline containing ^{14}C-mannitol (0.12 mM; ~ 4.5×10^6 cpm). The bathing saline was acidified after 1 hr to drive off dissolved $^{14}CO_2$, which was collected on glass fiber filters soaked in hyamine hydroxide and counted by liquid scintillation. Since no ^{14}C activity was detectable (sensitivity of the method was 5.4×10^{-10} moles $^{14}CO_2$/hr), it was concluded that mannitol is not metabolized.

Calculations and Statistics

In order to compare the instantaneous I_{sc} with tracer fluxes measured at intervals, I_{sc} recordings were integrated using a planimeter (model L30M, Lasico, Los Angeles, Calif.). Values are means ± standard errors unless stated otherwise; n = number of recta. Significant difference was determined using paired or unpaired t-tests.

Results

Verification of Active Cl Transport

Rectal I_{sc} indicated net transport of anions to the serosal side (or cations to the mucosal side) and declined exponentially after dissection ($t_{1/2}$ = 40 min), approaching a steady-state condition after 3 hr (Fig. 1*a*), in reasonable agreement with previous studies which utilized somewhat different preparations and salines [9, 31, 34]. J^{Cl}_{net} paralleled I_{sc} during the 9-hr experimental period. The discrepancy of 1 μeq cm^{-2} hr^{-1} between I_{sc} and J^{Cl}_{net} in unstimulated recta results from the transport of some ion which has not yet been identified [34] and is not due to inter-tissue variability since I_{sc}, V_t and R_t were virtually identical during forward and back flux measurements (i.e. differences between mean values were not significant at any sample time; $P \gg 0.2$). The mucosal side was positive with respect to the serosal side when recta were bathed in normal saline. R_t increased, then remained constant for several hours (Fig. 1*d*). The important finding is that no spontaneous increases in I_{sc}, Cl fluxes, V_t or R_t occur *in vitro* after the initial decay in Cl transport (Fig. 1*a–d*). An approximate steady-state condition is maintained between 4 and 8 hr after dissecting recta from locusts. Experiments were performed during this period unless otherwise indicated.

Table 2 shows the effects of adding 1 mM cAMP to the serosal side on Cl fluxes, I_{sc}, V_t and R_t under I_{sc} conditions. Recall that all tracer experiments were preceded by a 4-hr equilibration period, i.e. fluxes were measured between the 4th and 8th hour *in vitro*. Serosal addition of 1 mM cAMP caused 10-fold increases in J^{Cl}_{ms} and I_{sc}, a fivefold increase in V_t, and a small but significant increase in J^{Cl}_{sm} from 1.47 ± 0.14 to 1.77 ± 0.24 μeq cm^{-2} hr^{-1} (Table 2). R_t declined by about 50%. Maximal stimulation was

Table 2. Effects of cAMP on ^{36}Cl fluxes under I_{sc} conditions[a]

Saline		I_{sc} $\mu eq\ cm^{-2}\ hr^{-1}$	J_{ms}^{Cl} $\mu eq\ cm^{-2}\ hr^{-1}$	J_{sm}^{Cl} $\mu eq\ cm^{-2}\ hr^{-1}$	$\bar{x}\ J_{net}^{Cl}$ $\mu eq\ cm^{-2}\ hr^{-1}$
Normal					
	Control	1.42 ±0.19	2.32 ±0.22	1.47 ±0.14	0.85
	1 mM cAMP	10.72[b] ±1.06	11.23[b] ±1.51	1.77[c] ±0.24	9.46
Na-free					
	Control	1.94 ±0.20	2.80 ±0.17	2.70 ±0.27	0.1
	1 mM cAMP	8.11[b] ±0.44	7.76[b] ±0.57	2.27[c] ±0.31	5.49
HCO_3-free					
	Control	0.95 ±0.15	2.10 ±0.13	1.41 ±0.24	0.60
	1 mM cAMP	8.24[b] ±0.53	9.06[b] ±0.44	1.90[c] ±0.17	7.16
K-free					
	Control	1.17 ±0.26	1.72 ±0.15	1.68 ±0.31	0.04
	1 mM cAMP	3.24 ±0.60	3.58 ±0.53	2.41 ±0.50	1.17

[a] Means ± SE; n = 12 to 20 recta (I_{sc}), n = 6 to 10 (J_{ms}^{Cl}, J_{sm}^{Cl}).
[b] $P < 0.01$. cAMP was present for 60 to 90 min.
[c] $P < 0.05$.

obtained with about 1 mM cAMP, a higher dose than used previously (0.3 mM; [31]). Responsiveness of recta to cAMP was independent of time; I_{sc} increased to the normal stimulated value of 10 $\mu eq\ cm^{-2}\ hr^{-1}$ whether cAMP was added 2 or 6 hr following dissection. Control experiments also demonstrated that there was no difference between the effects of cAMP on recta obtained from male or female locusts.

Electrogenicity of Cl transport during cAMP stimulation was demonstrated directly by replacing Cl with anions which are not known to be transported in other tissues. Figure 2 shows that cAMP-stimulated I_{sc} is reduced by 91% when Cl is replaced with gluconate, consistent with previous results using different salines and noncompensating voltage clamps [30, 31, 34]. As usual, a small residual I_{sc} (~1 $\mu eq\ cm^{-2}\ hr^{-1}$) was insensitive to Cl replacement during cAMP exposure. Nevertheless, all of the stimulation of short-circuit current ($^{\Delta}I_{sc}$) could be accounted for by the increase in net Cl flux ($^{\Delta}J_{net}^{Cl}$), and this is true of all conditions employed in this study.

In a third series of experiments, V_t and unidirectional Cl fluxes were measured under open-circuit conditions and the observed flux ratios were compared with those calculated using the Ussing flux ratio equation by assuming that Cl crosses the rectal wall by simple diffusion. Cyclic-AMP increased V_t and J_{ms}^{Cl} whereas J_{sm}^{Cl} was similar during cAMP exposure under open-circuit and short-circuit conditions (1.34 ± 0.06 $\mu eq\ cm^{-2}\ hr^{-1}$ and 1.71 ± 0.12 $\mu eq\ cm^{-2}\ hr^{-1}$, respectively); however, J_{ms}^{Cl} was significantly lower under open-circuit conditions (4.75 ± 0.7 $\mu eq\ cm^{-2}\ hr^{-1}$ *vs.* 11.04 ± 0.64 $\mu eq\ cm^{-2}\ hr^{-1}$; $P < 0.001$). Importantly, ^{36}Cl flux ratios were always eight- to 10-fold higher during stimulation than those expected from the flux ratio equation.

In summary, Cl absorption across locust rectum fulfills classical criteria for active transport: J_{net}^{Cl} is maintained in the absence of electrical and chemical gradients and flux ratios at open-circuit are an order of magnitude higher than those predicted for simple diffusion. Sensitivity of cAMP-stimulated I_{sc} to anion substitutions and the good agreement between I_{sc} and J_{net}^{Cl} after cAMP addition indicate that Cl transport is the predominant electrogenic process in this epithelium.

Exchange Diffusion

The presence of a large, electrogenic Cl flux across locust rectum does not preclude some exchange diffusion and exchange diffusion has been proposed in

several epithelia from other invertebrates (reviewed in [15]). If J_{sm}^{Cl} occurs partly by exchange for mucosal Cl, then removing mucosal [Cl] should reduce J_{sm}^{Cl} (*see* e.g. [2], [18]). In contrast, we found that mean J_{sm}^{Cl} did not decline, but actually increased slightly from 0.62 ± 0.1 to 0.83 ± 0.15 μeq cm^{-2} hr^{-1}, under I_{sc} conditions when all mucosal Cl was replaced with methylsulfate ($\bar{x}$ ± SE, n = 6; difference not significant at $P > 0.1$). This suggests that J_{sm}^{Cl} is not stimulated by Cl on the "trans" side. Another expected result of exchange diffusion is that estimates of transepithelial Cl permeability (P_{Cl}) calculated from J_{sm}^{Cl} under I_{sc} conditions should not agree with those calculated under open-circuit conditions because electroneutral exchange flux would be insensitive to V_t. However, P_{Cl} was similar under short-circuit and open-circuit conditions (4.1×10^{-6} *vs.* 6.0×10^{-6} cm sec^{-1}). In short, these results are consistent with the notion that J_{sm}^{Cl} is passive and if Cl exchange diffusion is present in locust rectal epithelium, it occurs at a low rate that does not contribute significantly to ^{36}Cl fluxes under I_{sc} conditions.

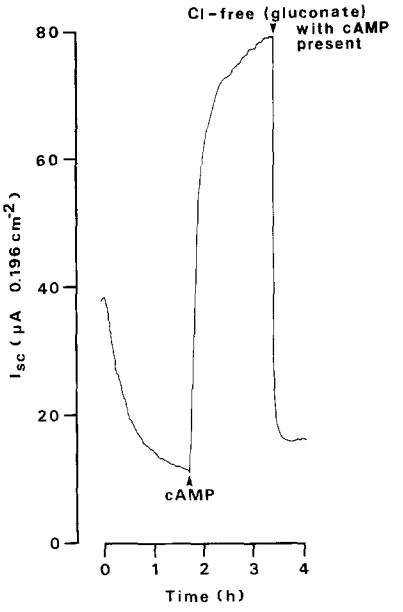

Fig. 2. Chloride dependence of cAMP-stimulated I_{sc}. Time 0 indicates time after dissection. At the first arrow, 1 mM cAMP was added to the serosal side while the rectum was bathed in normal saline. All Cl was replaced bilaterally with gluconate at the second arrow; 1 mM cAMP was still present on the serosal side

Selectivity

Recta were equilibrated under I_{sc} conditions and rinsed for 4 hr with saline in which Cl was completely replaced by other anions. I_{sc} was measured before and after addition of 1 mM cAMP to the serosal side. Cyclic-AMP stimulated I_{sc} only when Cl or Br were present (Fig. 3). The selectivity sequence in order of decreasing $^{\Delta}I_{sc}$ (μeq cm^{-2} hr^{-1}), was Cl (5.4) > Br (2.7) > PO_4, HPO_4 (0.5) > I, acetate, thiocyanate, SO_4, NO_3, F (all less than 0.2). Urate did not sustain I_{sc}. These data suggest that Cl is the principal anion transported by this system *in vitro*.

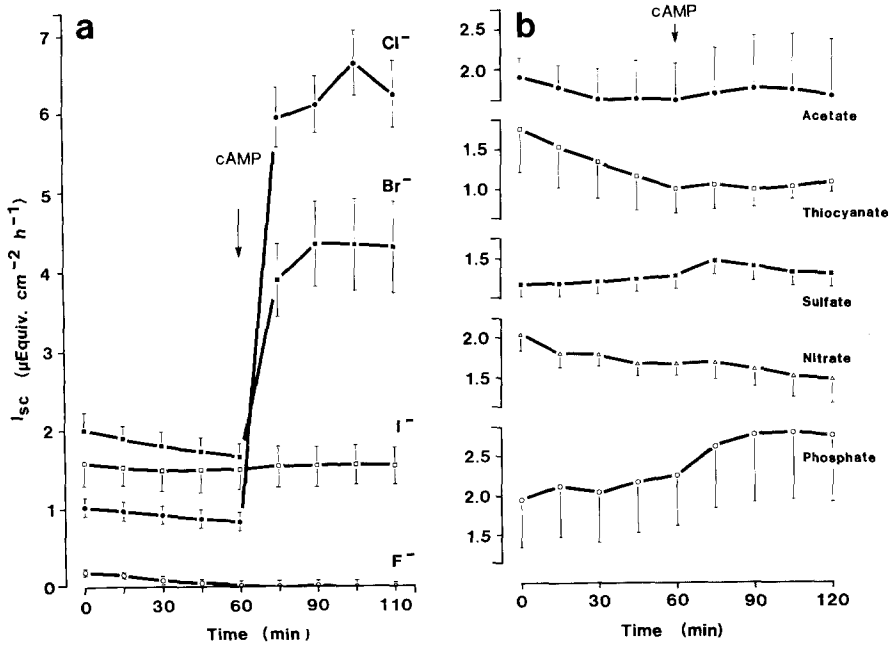

Fig. 3. Effect of 1 mM cAMP on I_{sc} in anion-substituted salines. Tissues were pre-equilibrated for 4 hr under I_{sc} conditions in saline in which all Cl was substituted with a test anion (*see* Table 1). Means ± SE; n = 5 – 6

Ionic Dependencies of Cl Transport

Sodium

To test whether Cl absorption across locust rectum requires external Na, tissues were equilibrated under I_{sc} conditions in normal saline as before and then exposed to 1 mM cAMP on the serosal side. Both chambers were rinsed thoroughly with nominally Na-free saline so that [Na] dropped from 114 mM to about 0.05 mM while cAMP (1 mM) was maintained on the serosal side. There was no significant change in Cl-dependent I_{sc} within the first 75 min ($P \gg 0.2$: I_{sc} declined from 16.0 ± 2.0 μeq cm^{-2} hr^{-1} to 14.6 ± 1.9 μeq cm^{-2} hr^{-1} ($\bar{x}$ ± SE, $n = 6$; for time course *see* ref. [15]) indicating that exogenous Na is not required for active Cl transport, at least during the first 75 min.

To ensure that the cAMP-stimulated I_{sc} reflects an increase in net Cl absorption in the virtual absence of Na, ^{36}Cl fluxes were measured after prolonged exposure to nominally Na-free saline before and after serosal addition of 1 mM cAMP. Recta were equilibrated for 4 hr in nominally Na-free saline and were rinsed at least three times with fresh saline to remove Na which had leached from the tissue. Final mucosal [Na] ranged from <1 to 200 μM. In nominally Na-free saline, 1 mM cAMP stimulated I_{sc} and J_{net}^{Cl} by 6.17 ± 0.37 and 5.48 μeq cm^{-2} hr^{-1}, respectively (Table 2). Despite the apparent difference between mean stimulated values of J_{net}^{Cl} and I_{sc} in normal and nominally Na-free saline, this disparity was not statistically significant at $P > 0.1$ and will be discussed below. It should be emphasized that electrical parameters were similar in tissues used for forward- and back-flux measurements: For example, in these experiments I_{sc} was 7.62 ± 0.47 *vs.* 8.86 ± 0.8 μeq cm^{-2} hr^{-1}, V_t was 27.9 ± 0.23 *vs.* 31.29 ± 3.01 mV and R_t was 136.78 ± 7.89 *vs.* 135.51 ± 11.81 Ωcm^2 during $m \rightarrow s$ and $s \rightarrow m$ flux experiments, respectively, and electrical data were pooled.

We considered whether Na-coupled Cl transport might continue to function because of trace amounts of sodium in nominally Na-free saline [28]. Such a mechanism would require extremely high affinity for Na and one would expect a correlation between the amount of Na contamination and I_{sc}.

Figure 4 shows that there was no correlation between cAMP-stimulated I_{sc} (which remained high) and trace levels of Na measured at the end of experiments. In several preparations, Na could not be detected (<1 μM) at the end when [Na] was usually highest. Less than 3% of the variation in I_{sc} may be attributed to [Na] ($r^2 = 0.0288$). Moreover, the K_t and V_{max} for net ^{22}Na flux across cAMP-stimulated, short-circuited recta in control saline are 18 mM and about 1 μeq cm^{-2} hr^{-1}, respectively (Black & Phillips, *unpublished observation*).

Some reduction in average J_{net}^{Cl} after long exposures (>5.5 hr) to Na-free saline is to be expected because of reduced Na-dependent amino acid absorption, which has been demonstrated in this tissue [1]. Consistent with this interpretation, saline containing 10 mM glucose and normal Na (114 mM) but lacking amino acids also did not sustain the I_{sc} and both Na-free and amino acid-free salines (with 110 mM Na) produced significantly higher ^{36}Cl backfluxes (two- to fivefold) and lower R_t. In view of the importance of amino acids, particularly proline [3] in maintaining Cl transport, some gradual reduction in active Cl transport probably results from metabolic decline. The more compelling observation is that a large J_{net}^{Cl} (6.0 μeq cm^{-2} hr^{-1}) is still observed after a 5.5-hr exposure to nominally Na-free saline, a condition which abolishes J_{net}^{Cl} and the "uphill" Cl electrochemical gradient across the mucosal border in epithelia where NaCl coentry is postulated (*see* [7]).

If Cl entry across the apical membrane were coupled to that of Na, the large stimulation of Cl unidirectional influx by cAMP should be paralleled by similar influx of Na. Figure 5*a* shows the time course of ^{36}Cl accumulation by rectal tissue when tied as a sac. Variation between preparations was reduced, but not eliminated by correcting for extracellular tracers using ^{3}H-mannitol as described in Materials and Methods. Control experiments showed that no time-dependent changes in extracellular volume occurred. In some experiments influx data had *Y*-intercepts below zero, suggesting a systematic overestimate of extracellular space. Unfor-

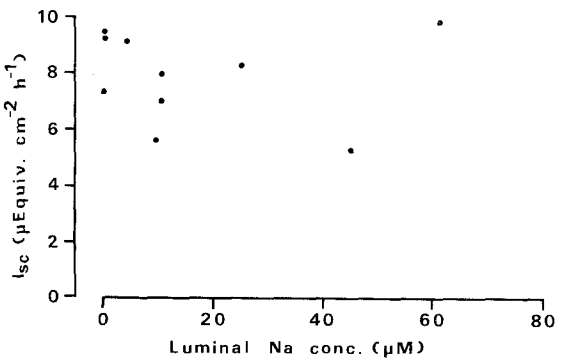

Fig. 4. Relationship between I_{sc} and trace level of Na in the mucosal half-chamber during Na-free experiments. Recta were exposed to nominally Na-free saline for a total of 7 to 8 hr. Cyclic-AMP (1 mM) was present on the serosal side during the last 2 hr. Each value is for a different rectal preparation

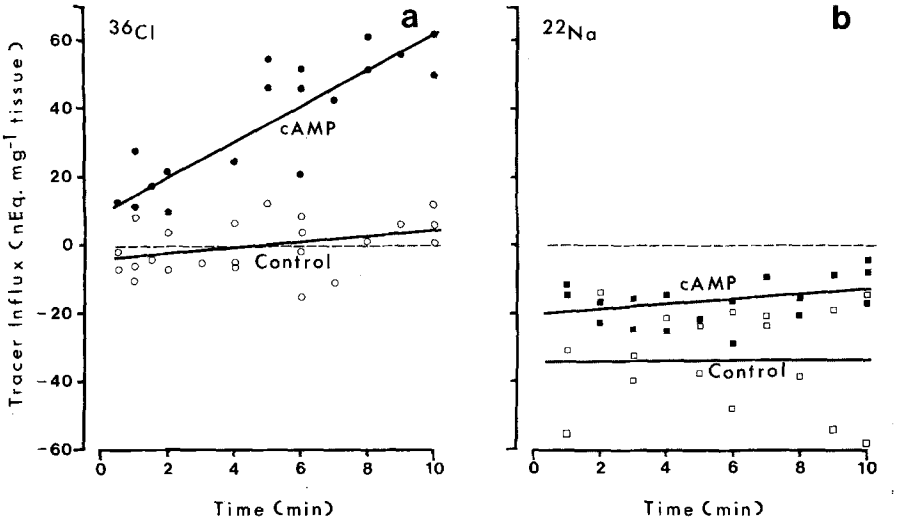

Fig. 5. Effects of cAMP on unidirectional influxes of ^{36}Cl and ^{22}Na into rectal tissue from the mucosal side. Initial influx of ^{36}Cl (*a*) and ^{22}Na (*b*) into tissue of everted rectal sacs was measured under control conditions (○, □) and after injection with 10 mM cAMP (●, ■). Each point is the result obtained from one animal. Tissue wet wet: 3 to 6 mg each. Nonzero intercepts in (*b*) indicate some overestimate of extracellular space. The difference between control and cAMP-stimulated influx of ^{36}Cl (i.e. slopes of regression lines) is significant at $P < 0.05$. In contrast, ^{22}Na influx is not different after exposure to cAMP ($P \gg 0.2$)

tunately, inulin could not be used as an extracellular space marker because it does not penetrate the layer of cuticle covering the mucosal surface of the epithelium.

Injection of 10 mM cAMP into sacs resulted in higher unidirectional influx of ^{36}Cl than in unstimulated controls (slope of 4.92 ± 1.57 *vs.* 0.83 ± 1.01 neq mg^{-1} min^{-1}; $\bar{x}$ ± SE, $P < 0.05$; 1 mg tissue is equivalent to ~7 mm^2 macroscopic area). V_t also increased after stimulation from 15.5 ± 1.1 to 58.9 ± 2.1 mV ($\bar{x}$ ± SE, n = 18 to 23). A higher [cAMP] was required to stimulate V_t maximally in sacs than was necessary using a flat sheet preparation. This may reflect more rapid degradation of the nucleotide by rectal tissue in the sac since it contains only a small volume of fluid (20 μl *vs.* 5 ml in the Ussing chamber).

The stimulation of unidirectional ^{36}Cl influx into rectal tissue during exposure to cAMP (from 0.7 to 4.2 μeq cm^{-2} hr^{-1}) was similar to the elevation of J^{Cl}_{ms} measured in Ussing chambers (from 1.52 ± 0.16 to 5.65 ± 0.77 μeq cm^{-2} hr^{-1}). In contrast, ^{22}Na influxes were low (0 to 0.8 μeq cm^{-2} hr^{-1}; Fig. 5*b*) and were not changed by exposure to 10 mM cAMP ($P \gg 0.2$). This lack of correlation between influxes is not consistent with NaCl coentry across the apical membrane and also suggests that electrical coupling between active Cl absorption and Na influx is minimal since ^{22}Na influx was not enhanced by the favorable V_t during cAMP stimulation. Under I_{sc} conditions, the rate of chloride absorption is 10-fold larger than that of K, however J^{K}_{net} equals J^{Cl}_{net} under open-circuit conditions [15]. Thus potassium is the principal counter-ion accompanying electrogenic Cl transport across locust rectum, even when the [Na] of the saline is much higher than that of K (114 mM Na, 10 mM K).

Table 3 shows that the addition of 1 mM ouabain to both sides for 1 hr does not alter J^{Cl}_{ms} in unstimulated recta or the subsequent stimulation by cAMP of I_{sc} and J^{Cl}_{net}. In fact, ouabain caused a small but significant increase in unstimulated I_{sc} from 1.5 ± 0.3 to 1.84 ± 0.21 μeq cm^{-2} hr^{-1} ($P < 0.01$), and J^{Cl}_{ms} showed a similar increase. In two preparations, addition of 1 mM cAMP produced stimulations of 8.33 and 4.83 μeq cm^{-2} hr^{-1} after exposure to ouabain for 5 hr at 22°C and 10 mM K. In summary, Cl transport appears relatively insensitive to ouabain in contrast to those vertebrate epithelia where Na-dependent Cl transport has been demonstrated [7].

Bicarbonate

Although perfectly HCO_3-free conditions cannot be ensured in intact tissues producing CO_2, cell membranes are highly permeable to CO_2 and removal of exogenous HCO_3 and CO_2 should eventually deplete cells of most HCO_3, H_2CO_3 and CO_2 due to efflux of these species down their enlarged electrochemical gradients. Prolonged exposure (6 hr) to nominally HCO_3- and CO_2-free saline had no effect on I_{sc}, unidirectional Cl fluxes, R_t, or V_t across locust recta (Table 2).

Table 3. Effects of sequential addition of 1 mM ouabain and 1 mM cAMP on transepithelial Cl fluxes[a]

	I_{sc} (μeq cm^{-2} hr^{-1})	J_{ms}^{Cl} (μeq cm^{-2} hr^{-1})	J_{sm}^{Cl} (μeq cm^{-2} hr^{-1})	J_{net}^{Cl} (μeq cm^{-2} hr^{-1})
Control	1.51	1.65	1.10	
	±0.13	±0.08	±0.07	0.55
	(48;12)	(24;6)	(24;6)	
1 mM ouabain	1.84[b]	1.73	1.32[b]	0.41
(1 hr)	0.21	0.08	0.07	
	(36;12)	(18;6)	(18;6)	
Ouabain (2 hr)	9.50[c]	9.1[c]	1.73[c]	7.37
+ 1 mM cAMP	±0.54	±0.51	±0.12	
	(36;12)	(18;6)	(18;6)	

[a] Mean + SE (number of flux periods; number of animals).
[b] Difference significant at $P < 0.05$.
[c] At $P < 0.01$.

Potassium

The effects of K were of particular interest since many insect epithelia normally reabsorb ions and water from a KCl-rich, low-Na fluid *in vivo*. Recall that primary urine secreted by the locust Malpighian tubules contains ~140 mM K when it enters the rectal lumen as compared with 10 mM K in the hemolymph (Table 1).

After 4 hr pre-equilibration in K-free saline, I_{sc}, J_{ms}^{Cl} and V_t in unstimulated recta were similar to those bathed in normal saline (Table 2). R_t was 25% higher under K-free conditions, suggesting that K contributes significantly to transepithelial conductance. Importantly, 1 mM cAMP caused only small stimulations in J_{net}^{Cl}, I_{sc}, V_t and R_t under these nominally K-free conditions. When 10 mM K was restored to both sides of cAMP-treated tissues (i.e. same concentration as normal saline), I_{sc}, J_{ms}^{Cl} and V_t increased and R_t declined to normal stimulated levels.

There is clearly a strong dependence of cAMP-stimulated Cl transport on exogenous K. Approximately 70% of the J_{net}^{Cl} under I_{sc} conditions is K-dependent. The nature of this K-requirement will be examined in more detail using ion-sensitive microelectrodes and electrophysiological techniques in the following paper.

Divalent Cations

Omitting Mg from normal saline did not alter cAMP-stimulated I_{sc} significantly during the first hour (8.67 ± 0.45 and 9.01 ± 0.46 μeq cm^{-2} hr^{-1} before and after removal, respectively). Similarly, when external Ca was removed for 1 hr there was no detectable change in cAMP-stimulated I_{sc} (9.44 ± 0.21 before and 10.13 ± 0.21 μeq cm^{-2} hr^{-1} after removal, respectively). To ensure that external Ca levels were $<10^{-7}$ M, 5 mM EGTA was also added to nominally Ca-free saline. EGTA addition had no effect on I_{sc} within 30 min. The effects of longer exposures to Ca-free saline + EGTA were not investigated. The lack of inhibition within 1 hr suggests that Cl transport across rectal tissue *per se* does not involve exogenous Ca or Mg.

Discussion

Comparison with Vertebrate Systems

The results described above suggest that the mechanism of Cl absorption across locust rectum is substantially different from models proposed for other epithelia. No significant inhibition of I_{sc} occurred during the first 75 min after replacing normal saline (~114 mM Na) with nominally Na-free saline, and large, cAMP-stimulated I_{sc} and J_{net}^{Cl} were observed during prolonged exposure to nominally Na-free saline. Although incubation in nominally Na-free saline for periods >6 hr depressed J_{net}^{Cl}, this effect was variable and the reduction was not statistically significant at the $P < 0.1$ level. Also, the decline in J_{net}^{Cl} was partly the result of a twofold increase in J_{sm}^{Cl}. As mentioned above, some reduction in active transport should be expected in Na-free saline since Na-coupled amino acid absorption has been demonstrated in locust rectum [1] and amino acids are necessary as respiratory substrates to maintain active Cl transport at normal levels. Na removal could also have other side-effects such as interfering with intracellular pH or Ca regulation. In summary, the finding that J_{net}^{Cl} was partially reduced by the rather

drastic procedure of removing Na for > 6 hr is not surprising. The more important observation is that a large J_{net}^{Cl} (6 μeq cm^{-2} hr^{-1}) persists under these conditions.

Sodium usually leached from the tissue during tracer flux experiments in Na-free saline, thereby increasing the mean [Na] of the mucosal half-chamber from 53.9 ± 12.2 to 94.6 ± 47.3 μM after 3.5 hr. Several preparations had final [Na] < 1 μM. At these low Na levels, NaCl coentry would require an unrealistically high "turnover" rate of Na at the mucosal border. Also, the slow leakage of Na into the mucosal solution alone suggests that the electrochemical gradient for Na under these conditions favors Na efflux across the apical membrane rather than coentry with Cl.

In order to account for the equivalence of I_{sc} and J_{net}^{Cl}, Na entering with Cl would have to be recycled back to the mucosal side by an apical Na pump or paracellularly through permselective junctions. There is no evidence for an apical Na pump: Na/K ATPase is seen on the basal but not apical membrane in dragonfly recta [17], and Na-sensitive microelectrode data also suggest that there is a Na/K exchange pump on the basal membrane [12]. It is also unlikely that Na is recycled paracellularly: From the dimensions of locust rectal cells, [Na], and I_{sc}, we calculate that a driving force equivalent to 2.5 to 25 volts would be needed between the lateral intercellular spaces and the mucosal side to cause paracellular diffusion at the required rate.

No correlation was observed between unidirectional influxes of ^{22}Na and ^{36}Cl into rectal tissue. These experiments were technically difficult because of small tissue size and presence of a large unstirred compartment on the mucosal side between the apical membrane and cuticle. However, the fact that Cl transport is quickly and reversibly blocked by lowering mucosal pH within the physiological range permitted tissues to be incubated at low pH to preequilibrate ^{36}Cl and ^{22}Na in the subcuticular space. ^{3}H-mannitol space was used to correct for extracellular tracers, although the data shown in Fig. 5 indicate that this correction was only partly successful. Only the rate of tracer entry (i.e. slope of the regression line) was used in calculations. The sixfold stimulation of ^{36}Cl uptake by cAMP suggests that tracer uptake is a reasonable indicator of unidirectional influx. When 10 mM cAMP was injected into the sac, influx of ^{22}Na did not change ($P > 0.2$; Fig. 5). The lack of correlation between ^{36}Cl and ^{22}Na influxes is in contrast to results obtained in several vertebrate epithelia where NaCl cotransport is postulated, and suggests that their movements are neither chemically nor electrically coupled. Also, Spring and Phillips [31] found that cAMP had no effect on transrectal ^{22}Na fluxes across locust rectum.

Further evidence for Na-independence is the insensitivity of locust Cl transport to known inhibitors of NaCl cotransport in other systems. In principal, the effects of inhibitors do not establish the presence or absence of transport mechanisms, since a particular mechanism might have variable sensitivity to inhibitors in different tissues, but they do provide circumstantial evidence against known mechanisms. Locust and other insect rectal tissues do contain a ouabain-sensitive Na/K ATPase that exhibits normal sensitivity to ouabain ($K_i = 10^{-6}$ M; [24]) although the potency of this inhibitor in intact tissues is controversial. Ouabain had no effect on locust rectal Cl transport, unlike Na-coupled systems in vertebrate animals. The diuretic furosemide is a well-known inhibitor of NaCl and Na, K, 2Cl cotransport systems; however, Cl-dependent I_{sc} in locust rectum is not affected by 1 mM furosemide after a 75-min exposure on both sides ($P \gg 0.2$; [15]). There was no obvious barrier to prevent access of furosemide to the apical membrane because the rectal cuticle was cut. Insensitivity to furosemide contrasts with all Na-coupled Cl transport systems which have been described to date.

Cl transport was not affected by removal of exogenous CO_2 and HCO_3 (Table 2). Intracellular levels of HCO_3 and CO_2 should be greatly reduced under these conditions as suggested by other workers using Cl-sensitive liquid-ion exchanger-type microelectrodes. For example, Garcia-Diaz and Armstrong [8] suggested that cytoplasmic [HCO_3] in *Necturus* gallbladder is <1.0 mM under these conditions. In nominally CO_2, HCO_3-free saline, intracellular HCO_3 must originate as metabolic CO_2. However, aerobic metabolism is apparently too low to provide sufficient CO_2 for hydration to HCO_3. Chamberlin [3] measured O_2 consumption of freely suspended rectal tissues (calculated $V_t < 3$ mV), and obtained a mean value of 2.8 μmol O_2 rectum^{-1} hr^{-1}. If we assume that the tissue has a maximal RQ of 1, and that 100% of the metabolically produced CO_2 could be converted to HCO_3 and made available at the mucosal side only, then the maximum supply of bicarbonate for 1:1 exchange with Cl is 3.2 μeq cm^{-2} hr^{-1} as compared to measured J_{ms}^{Cl} of 10 to 12 μeq cm^{-2} hr^{-1}. A stoichiometry of 3 Cl entering the cell for each HCO_3 seems unlikely given the normal range of intracellular HCO_3 concentrations in other epithelial cells (7.6 to 20.2 mM; [16]). Assuming a cell-to-mucosa HCO_3 gradient of 20-to-10 mM and membrane potential = −58 mV (measured under I_{sc} conditions), HCO_3 efflux would provide a driving force of only 76 mV compared to a total of 138 mV opposing the entry of 3 Cl. This

scenario would also require the direction of all HCO_3 to the apical side of the cell and perfectly efficient CO_2 hydration presumably catalyzed by carbonic anhydrase. We found that the carbonic anhydrase inhibitor acetazolamide had no effect on rectal Cl transport as measured by I_{sc} nor did SITS [15], a potent inhibitor of Cl/HCO_3 exchange in other cells. It is noteworthy that some alkalinization of the mucosal side was observed in nominally HCO_3-free saline; however, titratable base appears at a much lower rate than Cl transport ($\leq$39% of I_{sc}), follows a different time course following cAMP addition (i.e. delayed by 10 to 15 min), and may reflect ammonia production rather than HCO_3 secretion.

In summary, we tested some of the major predictions which have been used to substantiate NaCl cotransport and Cl/HCO_3 exchange models in other epithelia and obtained either negative or quantitatively unfavorable results. While both these entry mechanisms are electrically silent, measurements of intracellular ion activities and potentials under I_{sc} in the following paper suggest Cl entry is electrogenic and that under some experimental conditions the apical Na and K electrochemical gradients are too small to support the (measured) active entry of Cl.

Potassium Dependence of Cl Transport

The major fraction of Cl absorption across locust rectum requires exogenous K. Following prolonged exposure to nominally K-free saline, 1 mM cAMP caused only 35% of the normal stimulation of J_{net}^{Cl} and I_{sc}. Experiments in a later paper will describe the sidedness, selectivity, and concentration dependence of K stimulation but we obtained no evidence for a KCl cotransport mechanism.

The "K-insensitive" component of J_{net}^{Cl} is not due to imperfect K selectivity (i.e., Na substituting for K in K-free saline) because in control experiments, cAMP stimulated I_{sc} when both Na and K were substituted with N- methyl-D-glucamine, choline or tetramethyl ammonium ions.

These results provide evidence for an unusual Cl transport system in locust rectum which is not directly Na or HCO_3-coupled but which is stimulated by K. Like many insect epithelia, this tissue is normally bathed with fluid that contains K rather than Na as the predominant cation (e.g. Malpighian tubules, lepidopteran midgut, integument during moulting, salivary glands; [12, 24]). Cooper et al. [5] have shown that electrogenic Cl transport across the integument of *Manduca sexta* is also stimulated by K. The dependence on [K] is nearly identical to that in locust rectum ($K_a \simeq 5$ mM K) although the absolute rate of Cl transport is 10-fold lower. This new mechanism of Cl absorption may be widespread in insect epithelia that are normally bathed by K-rich fluid.

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