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Passive Sodium Movements Across the Opercular Epithelium: The Paracellular Shunt Pathway and Ionic Conductance

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Summary. The unidirectional Na^+, Cl^-, and urea fluxes across isolated opercular epithelia from seawater-adapted *Fundulus heteroclitus* were measured under different experimental conditions. The mean Na^+, Cl^-, and urea permeabilities were 9.30×10^{-6} cm · sec^{-1}, 1.24×10^{-6} cm · sec^{-1}, and 5.05×10^{-7} cm · sec^{-1}, respectively. The responses of the unidirectional Na^+ fluxes and the Cl^- influx (mucosa to serosa) to voltage clamping were characteristic of passively moving ions traversing only one rate-limiting barrier. The Na^+ conductance varied linearly with, and comprised a mean 54% of, the total tissue ionic conductance. The Cl^- influx and the urea fluxes were independent of the tissue conductance. Triaminopyrimidine (TAP) reduced the Na^+ fluxes and tissue conductance over 70%, while having no effect on the Cl^- influx or urea fluxes. Mucosal Na^+ substitution reduced the Na^+ permeability 60% and the tissue conductance 76%, but had no effect on the Cl^- influx or the urea fluxes. Both the Na^+ and Cl^- influxes were unaffected by respective serosal substitutions, indicating the lack of any Na^+/Na^+ and Cl^-/Cl^- exchange diffusion.

The results suggest that the unidirectional Na^+ fluxes are simple passive fluxes proceeding extracellularly (i.e., movement through a cation-selective paracellular shunt). This pathway is dependent on mucosal (external) Na^+, independent of serosal (internal) Na^+, and may be distinct from the transepithelial Cl^- and urea pathways.

Under short-circuited conditions, the isolated chloride cell-rich opercular epithelium of the seawater-adapted teleost, *Fundulus heteroclitus* (Burns & Copeland, 1950; Karnaky & Kinter, 1977), actively secretes Cl^- at a net rate equal to the applied short-circuit current (Karnaky, Degnan & Zadunaisky, 1977), with no measurable net Na^+ flux (Degnan, Karnaky & Zadunaisky, 1977). When bathed externally with seawater and internally with Ringer, the transepithelial potential difference is predominantly Na^+-sensitive and approximates the Na^+ equilibrium potential (Degnan & Zadunaisky, 1980*a*). In addition, the measured open-circuited Na^+ flux ratio agrees with that predicted with Ussing's (1960) equation, both before and after treatment with ouabain (Degnan & Zadunaisky, 1979), indicating that Na^+, K^+-ATPase is not directly involved in the transepithelial movements of this cation. Under short-circuited conditions, the Cl^- influx (mucosa to serosa) is unresponsive to agents known to influence the active Cl^- efflux (Degnan et al., 1977), while under open-circuited conditions, the Cl^- influx varies according to the changes in the transepithelial potential difference (Degnan & Zadunaisky, 1979).

Taken collectively, these findings provide evidence for concluding that the unidirectional Na^+ fluxes and the Cl^- influx behave passively in this isolated preparation. There is, however, an abundance of evidence with other chloride cell-containing epithelia, such as the marine teleost's gill epithelium, that Na^+ is actively secreted or exchanged for other cations and that Cl^- is exchanged for other anions (for a review, *see* Maetz & Bornancin, 1975). A more detailed study of the Na^+ and Cl^- movements across the opercular epithelium was therefore undertaken to better define the nature of the flux of these ions.

Materials and Methods

Tissue Preparation

Male and female killifish, *Fundulus heteroclitus*, were obtained from the Marine Biology Laboratory, Woods Hole, Mass., and adapted to artificial seawater (Utility Chemical Co., Paterson,

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N.J.). The dissection of the operculum has been described previously (Degnan et al., 1977). The Lucite chambers used in the present studies were of the type described previously for the operculum (Degnan & Zadunaisky, 1980*a*), which involved placing the tissue between two disks of polymerized Sylgard (Dow Corning, Midland, Mich.) with centrally located circular apertures. This technique avoids or minimizes edge damage (Helman & Miller, 1971). Two epithelia from the same fish were mounted in matching chambers and the experiments performed at room temperature (22–24 °C) during the months of August through November.

Electrical and Isotope Flux Measurements

The procedures for measuring the transepithelial potential difference ($\Delta\psi$), applying the short-circuit current (I_{sc}) or voltage-clamp current, and calculating the transepithelial conductance (G_T), have been described previously (Degnan et al., 1977). $^{22}Na^+$, $^{36}Cl^-$ and ^{14}C-urea were obtained from New England Nuclear, Boston, Mass. Concentrated isotope stock solutions were prepared in Ringer and 10–50 µl aliquots, containing 10 µCi of $^{22}Na^+$ or $^{36}Cl^-$ or 25 µCi of ^{14}C-urea, added to opposite sides of the epithelia in the matching chambers and allowed to equilibrate for 30–60 min. The Ringer used with the urea fluxes contained 5 mM urea. Samples from the "cold" side were taken at 30-min intervals and the activity determined by liquid scintillation. The "cold" side volume was kept constant by replacing the sample removed for counting with an equal volume of Ringer, or Ringer containing drug.

Solutions

The Ringer contained (in mM): NaCl, 135.0; KCl, 2.5; $MgCl_2$, 1.0; $CaCl_2$, 1.5; $NaHCO_3$, 16.0; glucose, 5.0; and was gassed with 95% O_2/5% CO_2 (pH 7.15). In the ion substitution experiments, Na^+ was replaced with equimolar amounts of choline and Cl^- was replaced with equimolar amounts of methylsulphate (Sigma Chemical Co., St. Louis, Mo.). The Na^+ and Cl^- concentrations of all solutions were confirmed by flame photometry and chloridometry, respectively. The change from an ion-rich to ion-free solution was accomplished by perfusion of the chamber half with enough ion-free solution until a complete turnover of chamber fluid was accomplished. This technique has been described previously (Degnan & Zadunaisky, 1980*a*), allows for the continual short-circuiting during solution changes, and avoids mechanical stress to the tissue resulting from hydrostatic pressure gradients.

Concentrated stock solutions of amiloride (Merck, Sharp & Dohme, West Point, Pa.) and amphotericin B (Fungizone, E.R. Squibb & Sons, Princeton, N.J.) were prepared in Ringer and 50 or 100 µl aliquots added to both sides of the chamber, which, when diluted into the half-chamber volume, gave a final concentration of 10^{-4} M. Concentrated stock solutions of TAP (2,4,6-triaminopyrimidine; Sigma Chem. Co., St. Louis, Mo.) were prepared in 0.2 N HCL and the pH and Cl^- concentration adjusted to that of the Ringer. 100–150 µl aliquots were added to both sides of the chamber, giving a final concentration of 10^{-2} M when diluted into the half-chamber volume.

Calculations

All values reported are for steady-state conditions only. Steady-states were defined as constant or slowly changing (<10%/hr) short-circuit or voltage-clamp currents. The unidirectional fluxes for each experiment were calculated as the average of two or more successive 30-min steady-state flux periods. The serosal to mucosal flux (J_{sm}, efflux) was measured in one preparation while the mucosal to serosal flux (J_{ms}, influx) was measured in the paired preparation. After the addition of drugs or the substitution of an ion, samples for flux measurements were taken at 30-min intervals after the establishment of new steady-state conditions. In the equations listed, Na^+ and Cl^- activities (Robinson & Stokes, 1959) rather than concentrations, were used in the calculations. The data are expressed as mean ± standard error of the mean (mean ± SEM) with the number of experiments given in parentheses. Statistical significance was taken at the level $P<0.01$.

Results

The Relationship between the Na^+ and Total Ionic Conductances

Under equilibrium conditions, the conductance of a pathway to a passively and independently moving ion, in this case Na^+, is given by the equation (Hodgkin, 1951):

$$G_{Na^+} = \frac{z^2 F^2}{RT} \cdot J^{Na^+}, \tag{1}$$

where G_{Na^+} is the Na^+ conductance, z is the valence, F is Faraday's constant, R is the gas constant, T is the absolute temperature, and J^{Na^+} is the measured unidirectional Na^+ flux. Under steady-state, short-circuited conditions, the total ionic conductance (G_T) is given by the ratio $I_{sc}/\Delta\psi$, and the Na^+ permeability (P_{Na^+}) can be approximated under a variety of conditions from the ratio $J^{Na^+}/[Na^+]$, where $[Na^+]$ is the Na^+ activity of the compartment from which the flux originates. By applying Eq. (1) to the Na^+ fluxes across the operculum, when bathed on both sides with identical solutions (Ringer) and short-circuited ($\Delta\psi=0$), an approximation of the G_{Na^+} was obtained and compared to the G_T. The means of these parameters and the P_{Na^+} are listed in Table 1. The G_{Na^+}/G_T ratio for all preparations ranged from 0.41 to 0.69, giving a combined mean of 0.54 ± 0.01, indicating that approximately 54% of the total ionic conductance was a Na^+ conductance. When $\Delta\psi=0$, this percentage represents the mean partial ionic conductance.

Initially, the relationship between the G_{Na^+} and G_T was analyzed by the method of least squares separately for the effluxes and influxes. Since the regression lines for the two sets of data did not differ with respect to slopes, elevations, and residual variances, the data were combined and fitted with a single regression line (Fig. 1). The result demonstrated a linear relationship between the G_T and the G_{Na^+}, which was described by the equation:

$$G_T = 1.72 G_{Na^+} + 0.62, \tag{2}$$

Table 1. Na^+ fluxes, permeabilities, conductances, and tissue ionic conductances across isolated short-circuited opercular epithelia from seawater-adapted *Fundulus heteroclitus*

	J^{Na^+} ($\mu eq \cdot cm^{-2} \cdot hr^{-1}$)	P_{Na^+} (10^{-6} cm $\cdot$ sec^{-1})	G_{Na^+} (mmho $\cdot$ cm^{-2})	G_T (mmho $\cdot$ cm^{-2})	G_{Na^+}/G_T
$J_{sm}^{Na^+}$ (25)	3.764 ± 0.345	8.68 ± 0.82	3.96 ± 0.36	8.0 ± 0.8	0.50 ± 0.01
$J_{ms}^{Na^+}$ (25)	4.297 ± 0.328	9.92 ± 0.80	4.52 ± 0.35	8.0 ± 0.6	0.58 ± 0.01
P	> 0.025	> 0.025	> 0.025	> 0.90	< 0.005

Data are steady-state measurements and calculations for paired epithelia from the same fish in which the efflux ($J_{sm}^{Na^+}$) was measured in one preparation and the influx ($J_{ms}^{Na^+}$) in the other. Each experiment represents the control for all experiments reported and consists of the average of a minimum of two successive steady-state 30-min flux periods. Data are expressed as mean ± SEM with the number of experiments in parentheses.

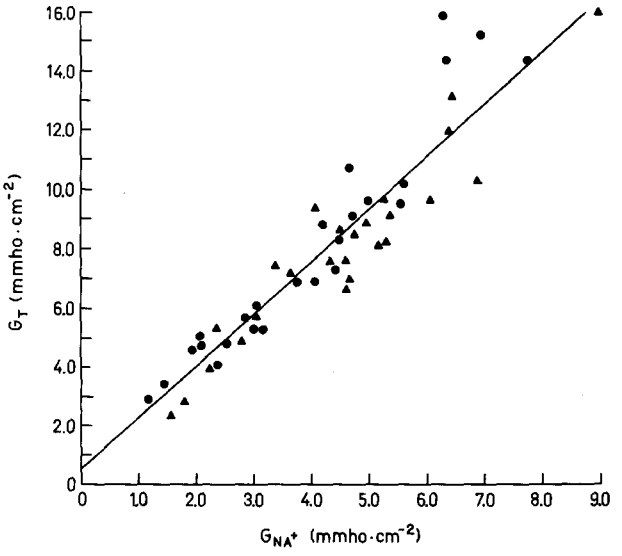

Fig. 1. The comparison of the partial Na^+ conductance and the total ionic conductance in isolated, short-circuited epithelia bathed on both sides with Ringer. Each point represents the average of a minimum of two successive steady-state flux periods (● = $J_{sm}^{Na^+}$; ▲ = $J_{ms}^{Na^+}$). The regression line was fitted by the method of least squares

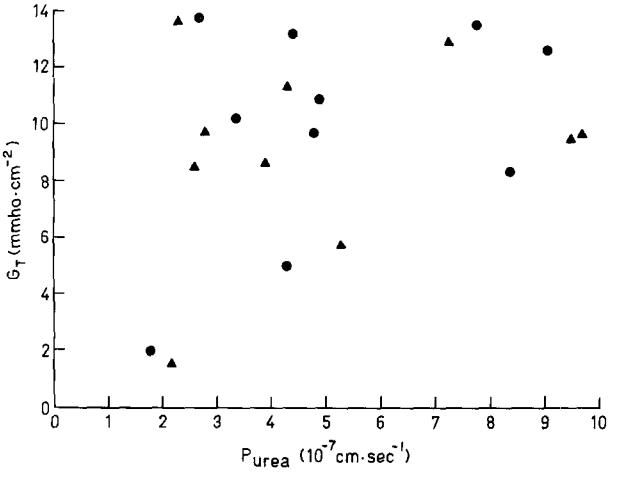

Fig. 2. The comparison of the urea permeability and the total ionic conductance in isolated, short-circuited epithelia bathed on both sides with Ringer. Specifications are as in Fig. 1 (● = J_{sm}^{urea}; ▲ = J_{ms}^{urea})

with a correlation coefficient of 0.94. In contrast, a similar comparison demonstrated an apparent independence between the G_T and the urea permeability (P_{urea}), as shown in Fig. 2. The mean urea efflux and influx were 0.009 ± 0.001 and 0.009 ± 0.002 $\mu eq \cdot cm^{-2} \cdot hr^{-1}$, respectively, resulting in no significant ($P > 0.90$) net urea flux ($n = 10$). The mean P_{urea}, calculated with the combined fluxes ($n = 20$), was 5.05 (± 0.58) $\times 10^{-7}$ cm $\cdot$ sec^{-1}, which was 5.4% of the combined mean P_{Na^+} of 9.30 (± 0.57) $\times 10^{-6}$ cm $\cdot$ sec^{-1} ($n = 50$).

Response of the Unidirectional Na^+ Fluxes to Voltage-Clamping

Mandel and Curran (1972) demonstrated that the passive movement of an ion through the extracellular pathway could be distinguished from that through the transcellular pathway by measuring the voltage dependency of the unidirectional fluxes. Starting with the Nernst-Planck equation, describing the net flux of an ion under the influence of an electrochemical gradient, and integrating across a uniform barrier with the constant field assumption of Goldman (1943), they arrived at the following equation for the net flux:

$$J_{net} = -\frac{D_i}{d} \cdot \frac{F \Delta\psi z_i}{RT} \cdot \left[\frac{C_i^o - C_i^i \exp(z_i F \Delta\psi / RT)}{1 - \exp(z_i F \Delta\psi / RT)}\right] \quad (3)$$

where D_i is the diffusion coefficient of the ion, d is the thickness of the barrier, C_i is the ionic activity inside and outside, $\Delta\psi$ is the potential across the membrane, and the other symbols are as in Eq. (1). Assuming the net flux arises from two independent

unidirectional fluxes, the equation for the unidirectional flux of Na^+, for example, can be expressed as:

$$J^{Na^+} = \frac{P_{Na^+} zF\Delta\psi/RT}{1-\exp(-zF\Delta\psi/RT)} \cdot C \quad (4)$$

where $P_{Na^+} = D_i/d$ is the Na^+ permeability and C the Na^+ activity of the compartment from which the flux originates.

If the Na^+ permeability is assumed to be independent of the voltage across the membrane, and the Na^+ activity of the compartment from which the flux originates kept constant, the relationship between the Na^+ unidirectional flux when the preparation is short-circuited ($\Delta\psi = 0$) and voltage-clamped ($\Delta\psi \neq 0$) is given by the following equation:

$$J^{Na^+}_{\Delta\psi \neq 0} = J^{Na^+}_{\Delta\psi = 0} \frac{zF\Delta\psi/RT}{1-\exp(-zF\Delta\psi/RT)}. \quad (5)$$

The relationship given by Eq. (5) was applied to the unidirectional Na^+ fluxes across the opercular epithelium and the results summarized in Table 2. For each flux direction (J_{sm} and J_{ms}), the short-circuited flux was measured, the flux at the clamped voltage (± 25 mV) predicted with Eq. (5), and compared to the measured fluxes at the clamped voltages. The results indicate no significant differences in the measured and predicted parameters and therefore allow these fluxes to be described as passive and traversing only one rate-limiting barrier. These agreements also support the assumption that the P_{Na^+} was voltage-independent within the applied voltage range of these experiments. This type of behavior is attributed to movement through the paracellular shunt pathway with its single tight junction providing the rate-limiting barrier (Mandel & Curran, 1972; Bruus, Kristensen & Larsen, 1976). Alternative explanations, such as a high Na^+ permeable membrane in series with a low Na^+ permeable membrane, are possible. Evidence in support of paracellular Na^+ movements across the opercular epithelium comes from the lack of effects of amiloride and amphotericin B (Table 3), two compounds known to influence transcellular Na^+ pathways (Bentley, 1968; Lichtenstein & Leaf, 1965). Although amiloride is only effective in "tight" epithelia, it was used because the opercular epithelium is not exclusively "leaky" and increases its transepithelial resistance 3–8 times *in vitro* in response to low external NaCl concentrations[1]. Amphotericin B, on the other hand, stimulates Na^+ transport in both "tight" (Lichtenstein & Leaf, 1965) and "leaky" (Graf & Giebisch, 1979) epithelia. The observed decrease in the $J^{Na^+}_{sm}$ across the opercular epithelium in response to amphotericin B can be attributed mostly to the reduction in the G_T for these preparations. This reduction was most likely brought about by the action of sodium deoxycholate, used to solubilize amphotericin B in the commercial preparation, Fungizone. In a series of experiments (unreported), pure sodium desoxycholate, at doses equal to that in a 10^{-4} M dose of amphotericin B, produced large reductions in the I_{sc} and G_T across the opercular epithelium.

[1] Degnan, K.J., Zadunaisky, J.A. The sodium and chloride dependency of active chloride secretion across the opercular epithelium *(submitted)*.

Table 2. Comparison of the measured and predicted Na^+ fluxes and flux ratios across isolated, voltage-clamped opercular epithelia of seawater-adapted *Fundulus heteroclitus*

Clamp voltage ($\Delta\psi$) (mV)	J^{Na^+} (μeq · cm^{-2} · hr^{-1})		P	$J^{Na^+}_{\Delta\psi=0}/J^{Na^+}_{\Delta\psi\neq 0}$		P
	Measured	Predicted		Measured	Predicted	
J_{sm} (5)						
−25.0	2.467 ± 0.258	2.540 ± 0.286	>0.60	0.560 ± 0.029	0.589	>0.30
0.0	4.313 ± 0.485					
+25.0	6.391 ± 0.601	6.767 ± 0.762	>0.40	1.459 ± 0.060	1.569	>0.10
J_{ms} (5)						
−25.0	7.896 ± 0.349	7.824 ± 0.770	>0.80	1.621 ± 0.102	1.569	>0.60
0.0	4.987 ± 0.491					
+25.0	2.725 ± 0.270	2.937 ± 0.289	>0.025	0.546 ± 0.014	0.589	>0.025

Each individual experiment consisted of a minimum of two successive steady-state 30-min flux periods at each clamp voltage. The clamp voltage ($\Delta\psi$) is expressed as serosa relative to mucosa. Other specifications are as in Table 1.

Table 3. The effects of amiloride and amphotericin B on the Na^+ fluxes and tissue conductances across isolated, short-circuited opercular epithelia from seawater-adapted *Fundulus heteroclitus*

	$J_{sm}^{Na^+}$	$J_{ms}^{Na^+}$	$G_T(sm)$	$G_T(ms)$
	($\mu eq \cdot cm^{-2} \cdot hr^{-1}$)		($mmho \cdot cm^{-2}$)	
Control (5)	4.352±1.053	4.881±1.048	9.1±2.1	9.0±2.0
10^{-4} M amiloride	4.219±1.061	4.816±1.100	8.5±2.3	8.2±1.9
Percent change	3.1	1.3	6.2	9.1
P	>0.50	>0.70	>0.20	>0.10
Control (5)	3.705±0.915	3.955±0.915	7.9±2.4	7.8±2.0
10^{-4} M amphotericin B	2.182±0.525	3.828±0.830	6.0±1.6	7.5±1.9
Percent change	41.1	3.2	24.0	3.8
P	>0.02	>0.50	>0.05	>0.60

The letters in parentheses after the conductances correspond to the flux measurements in that direction. Other specifications are as in Table 1.

The Effects of TAP on the Na^+ and Urea Fluxes and Tissue Conductances

TAP was initially reported to specifically block paracellular Na^+ movements and decrease the ionic conductance across the gallbladder (Moreno, 1975*a*). Subsequently, TAP has been reported to affect cellular cation pathways (Lewis & Diamond, 1976; Balaban, Mandel & Benos, 1979; Reuss & Grady, 1979) and active Cl^- transport (Frizzell, Clayton & Field, 1978) in addition to its paracellular effects. Nevertheless, TAP still can serve as a useful probe of paracellular permeability, particularly when other criteria demonstrate extracellular Na^+ movements. According to Moreno (1975*a*), TAP is most effective in its monoprotonated form, which has a pK of 6.7. Lowering the pH of the Ringer bathing both sides of the operculum to 6.5, by decreasing the HCO_3^- concentration at a fixed pCO_2, resulted in a large reduction in the G_T and lowered the Na^+ fluxes below levels necessary for reliable isotope measurements. Under these conditions, 10 mM TAP had no measurable effect on the Na^+ fluxes and tissue conductances. It was therefore decided to test TAP at the usual pH of 7.15. Moreno (1975*a*) showed that a 10 mM concentration of TAP at this pH contains 2.6 mM of the active monocationic form. The results of these experiments are summarized in Table 4. TAP reduced the Na^+ fluxes and tissue conductances over 70%. However, the G_{Na^+}/G_T ratio remained relatively constant around 0.50, indicating that TAP reduced the conductance of all ions proportionally. In similar experiments, TAP produced comparable reductions in the conductances while having no effect on the urea fluxes (Table 4). In addition, TAP also produced a significant ($P<0.001$) inhibition in the I_{sc} as shown in Fig. 3. In 20 preparations, 10 mM TAP inhibited the I_{sc} a mean 64.6%.

During these studies, a spontaneous reversal of the inhibitory effects of TAP on the Na^+ fluxes and G_T's were observed, while the I_{sc}'s remained relatively constant at their inhibited levels. This reversal usually occurred within 60–90 min after TAP and within the same time period in which an increase in the urea fluxes and G_T's were observed. This reversal steadily increased with time and probably reflected a generalized deterioration of the tissue.

The Effect of Unilateral Na^+ Substitutions on the Na^+ and Urea Fluxes

The effects of unilateral Na^+ substitutions on the unidirectional Na^+ and urea fluxes and tissue conductances are summarized in Table 5. Mucosal Na^+ substitution resulted in mean 60.0% and 76.2% reductions in the $J_{sm}^{Na^+}$ and G_T, respectively. Similar substitutions reduced the G_T a mean 70.4%, but had no effect on the J_{sm}^{urea}. On the other hand, serosal Na^+ substitution had no significant effect on the $J_{ms}^{Na^+}$, while decreasing the G_T a mean 57.9%. Similarly, serosal Na^+ substitution reduced the G_T a mean 54.0%, while having no effect on the J_{ms}^{urea}. The results demonstrated the sensitivity of the Na^+ pathway to mucosal Na^+ and the insensitivity to serosal Na^+. The magnitude of the reduction in the G_T in response to mucosal Na^+ substitution was greater than the mean partial Na^+ conductance of this tissue. The reduction in the G_T in response to serosal Na^+ substitution approximated the non-Na^+ conductance part of this tissue. This can be explained by a Na^+-dependent conductance of another ion(s). Previous studies demonstrated the bilateral Na^+-dependency of Cl^- secretion across the operculum (Degnan & Zadunaisky, 1980*b*). The effect of mucosal Na^+ substitution on the G_T can be explained by a reduction

Table 4. The effects of TAP on the Na^+ and urea fluxes and tissue conductances across isolated, short-circuited opercular epithelia from seawater-adapted *Fundulus heteroclitus*

	J_{sm}	J_{ms}	$G_T(sm)$	$G_T(ms)$
	($\mu eq \cdot cm^{-2} \cdot hr^{-1}$)		($mmho \cdot cm^{-2}$)	
Na^+ Fluxes				
Control (5)	3.406±1.040	5.201±1.578	7.4±2.3	10.4±3.6
10^{-2} M TAP	0.543±0.171	1.403±0.689	1.7±0.8	2.5±1.5
Percent change	84.1	73.3	77.0	76.0
P	>0.025	<0.01	<0.01	>0.02
Urea fluxes				
Control (5)	0.010±0.002	0.008±0.002	12.7±0.5	9.6±0.6
10^{-2} M TAP	0.011±0.002	0.009±0.002	4.6±1.1	2.7±0.6
Percent change	10.0	12.5	63.8	71.9
P	>0.90	>0.10	<0.005	<0.001

The letters in parentheses after the conductances correspond to the flux measurements in that direction. Other specifications are as in Table 1.

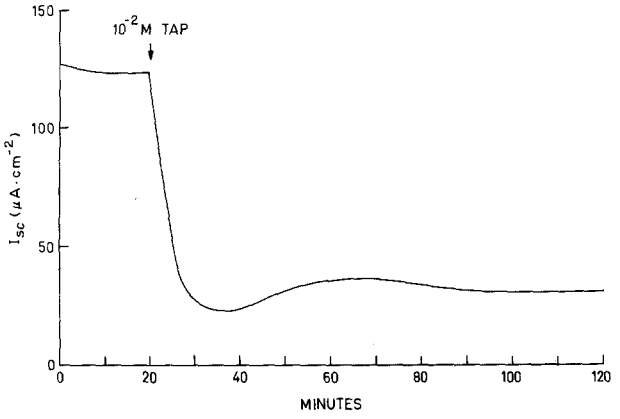

Fig. 3. The inhibitory effect of TAP (pH 7.15) on the I_{sc} across the opercular epithelium, typical of that observed in 20 preparations

in the G_{Na^+} of the shunt pathway and a reduction in the G_{Cl^-} of the mucosal membrane, while the effect of serosal Na^+ substitution can be attributed to a reduction in the G_{Cl^-} of the serosal membrane. The results also demonstrated the absence of a transepithelial Na^+/Na^+ exchange and suggested the possibility of a separate urea pathway.

Nature of the Cl^- Influx across the Opercular Epithelium

Investigations similar to those performed on the Na^+ and urea fluxes were also performed on the Cl^- influx, presumably a passive flux. A comparison of

Table 5. The effects of unilateral Na^+ substitutions on the unidirectional Na^+ and urea fluxes, tissue conductances, and permeabilities across isolated, short-circuited opercular epithelia from seawater-adapted *Fundulus heteroclitus*

	J^{Na^+}	J^{urea}	$G_T(Na^+)$	G_T(urea)	P_{Na^+}	P_{urea}
	($\mu eq \cdot cm^{-2} \cdot hr^{-1}$)		($mmho \cdot cm^{-2}$)		(10^{-6} cm · sec^{-1})	(10^{-7} cm · sec^{-1})
J_{sm} (5)						
Control	3.237±0.367	0.008±0.002	6.7±1.0	7.1±1.6	7.81±0.90	4.50±1.08
M $Na^+ = 0$	1.295±0.207	0.008±0.001	1.6±0.2	2.1±0.3	3.14±0.50	4.57±0.52
% change	60.0	0.0	76.2	70.4	59.8	1.6
P	<0.001	>0.90	<0.005	>0.02	<0.001	>0.90
J_{ms} (5)						
Control	3.935±0.506	0.009±0.003	7.6±0.6	8.7±2.3	9.53±1.22	5.24±1.41
S $Na^+ = 0$	3.305±0.329	0.012±0.003	3.2±0.4	4.0±1.0	8.00±0.80	6.81±1.71
% change	16.0	33.3	57.9	54.0	16.1	30.0
P	>0.20	>0.30	<0.001	>0.025	>0.20	>0.30

M = mucosa; *S* = serosa. Other specifications are as in Tables 1 and 3.

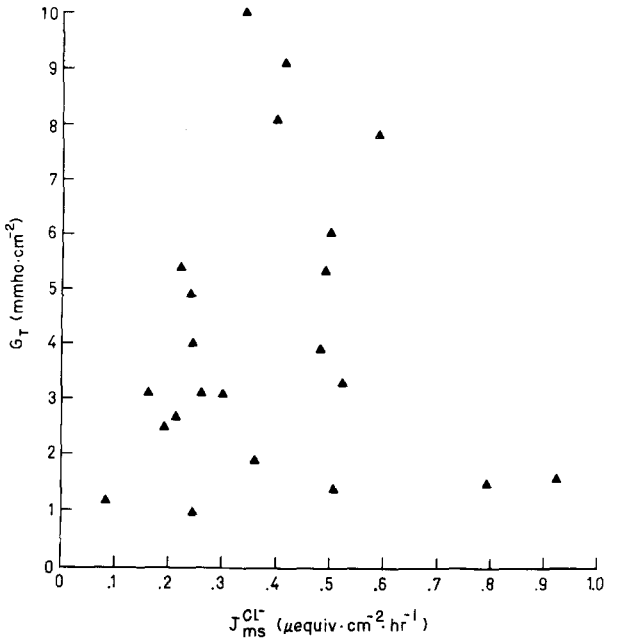

Fig. 4. The comparison of the Cl^- influx and the total ionic conductance in isolated, short-circuited epithelia bathed on both sides with Ringer. Specifications are as in Fig. 1

Table 7. The effects of TAP and unilateral Na^+ and Cl^- substitutions on the unidirectional Cl^- fluxes and tissue conductances across isolated, short-circuited opercular epithelia from seawater-adapted *Fundulus heteroclitus*

	Flux ($\mu eq \cdot cm^{-2} \cdot hr^{-1}$)	G_T ($mmho \cdot cm^{-2}$)
Control $J^{Cl^-}_{sm}$ (5)	6.187±0.734	9.9±1.3
TAP, 10^{-2} M	1.480±0.449	2.7±0.4
Percent change	76.1	72.9
P	<0.001	<0.001
Control $J^{Cl^-}_{ms}$ (6)	0.308±0.060	4.5±1.6
TAP, 10^{-2} M	0.340±0.095	2.5±0.8
Percent change	10.4	44.4
P	>0.50	>0.50
Control $J^{Cl^-}_{ms}$ (6)	0.527±0.116	3.3±0.6
Serosal $Cl^-=0$	0.522±0.091	2.7±0.6
Percent change	1.0	18.2
P	>0.90	>0.10
Control $J^{Cl^-}_{ms}$ (4)	0.384±0.086	4.8±0.6
Mucosal $Na^+=0$	0.366±0.086	1.9±0.4
Percent change	5.2	61.7
P	>0.50	<0.005

Specifications are as in Table 1.

the G_T and $J^{Cl^-}_{ms}$ for 22 control periods, with the operculum bathed on both sides with Ringer and short circuited, revealed no correlation between these two parameters (Fig. 4). A similar comparison between the I_{sc} and the $J^{Cl^-}_{ms}$ (unreported) demonstrated a similar lack of correlation, indicating that the $J^{Cl^-}_{ms}$ was independent of the G_T and I_{sc}. The mean P_{Cl^-} calculated from these experiments was 1.24 (±0.21) $\times 10^{-6}$ cm · sec^{-1}, which was 13% of the mean P_{Na^+}, and represented 22 (±6)% of the G_T for these experiments. Voltage-clamp Cl^- influx experiments indicated that this flux was a passive diffusional flux traversing only one rate-limiting barrier (Table 6). TAP (pH 7.15) had no effect on the $J^{Cl^-}_{ms}$, while reducing the G_T 44.4%, but did significantly inhibit the active $J^{Cl^-}_{sm}$ 76.1% (Table 7). Similar to the pattern observed with the Na^+ and urea fluxes, a spontaneous increase in both Cl^- fluxes and the G_T's was observed within the 60–90 min period after inhibition by TAP, while the I_{sc} remained inhibited. Serosal Cl^- and mucosal Na^+ substitutions had no effects on the $J^{Cl^-}_{ms}$ (Table 7), indicating that there was no transepithelial Cl^-/Cl^- exchange and that the $J^{Cl^-}_{ms}$, like the urea fluxes, was insensitive to changes in mucosal Na^+ concentrations.

The inhibitory effect of TAP on the active Cl^- efflux across the operculum was similar to the effect of TAP on the active Cl^- transport across the flounder intestine (Frizzell et al., 1978). The lack of effect of TAP on the passive $J^{Cl^-}_{ms}$ was consistent with the observations of Moreno (1975*a*), suggesting that this agent does not affect diffusional anion fluxes. The voltage-clamp studies suggested that the $J^{Cl^-}_{ms}$ was an extracellular flux, and the insensitivity of this flux to TAP and mucosal Na^+ substitution suggested an extracellular pathway separate from the Na^+ path-

Table 6. Comparison of the measured and predicted Cl^- influx and flux ratios across isolated, voltage-clamped opercular epithelia of seawater-adapted *Fundulus heteroclitus*

Clamp voltage ($\Delta\psi$) (mV)	$J^{Cl^-}_{ms}$ ($\mu eq \cdot cm^{-2} \cdot hr^{-1}$)		P	$J^{Cl^-}_{\Delta\psi=0}/J^{Cl^-}_{\Delta\psi\neq 0}$		P
	Measured	Predicted		Measured	Predicted	
−25.0	0.273±0.061	0.202±0.035	>0.10	0.701±0.073	0.589	>0.10
0.0	0.342±0.059					
+25.0	0.449±0.084	0.537±0.093	>0.10	1.336±0.138	1.569	>0.10

$N=6$. Other specifications are as in Tables 1 and 2.

way. In all respects, the $J_{ms}^{Cl^-}$ and urea fluxes were similar, suggesting that these may traverse the same pathway.

Discussion

Nature of the Na^+ and Cl^- Movements across the Opercular Epithelium

The present investigations support the findings of previous investigations demonstrating passive Na^+ behavior in this isolated preparation. In addition, they indicated the absence of a Na^+/Na^+ exchange diffusion and provided evidence to suggest that the bidirectional Na^+ fluxes are extracellular. The evidence favoring extracellular diffusion are the voltage-clamp data, indicating that the Na^+ pathway has characteristics of a single diffusional barrier (Mandel & Curran, 1972). In "leaky" epithelia this pathway is assumed to be the paracellular shunt with its single tight junction. These results, however, do not exclude the possibility of a single cellular rate-limiting barrier. Arguments against such a cellular pathway are the lack of effects of amiloride and amphotericin B, two compounds known to influence transcellular Na^+ movements (Bentley, 1968; Salako & Smith, 1970; Lichtenstein & Leaf, 1965), and the large inhibitory effect of TAP, a compound which blocks paracellular cation shunts (Moreno, 1975*a*). Unfortunately, subsequent investigations on a variety of epithelia have demonstrated multiple effects of these compounds. Amiloride inhibits Cl^- exchange diffusion (Kristensen, 1978), passive Cl^- diffusion (Candia, 1978), and paracellular Na^+ movements (Balaban et al., 1979) in amphibian skin. Amphotericin B increases the conductance of other small cations in the toad urinary bladder (Reuss, Gatzy & Finn, 1978) and *Necturus* gallbladder (Reuss, 1978). TAP reportedly has an amiloride-like effect on Na^+ transport in frog skin (Zeiske, 1975; Balaban et al., 1979), and decreases apical membrane K^+ conductance in *Necturus* gallbladder (Reuss & Grady, 1979).

Regardless of these multiple effects, neither amiloride nor amphotericin B has significant effects on the unidirectional Na^+ fluxes, suggesting that no significant transcellular Na^+ pathway exists across the opercular epithelium under these conditions. These drugs were tested bilaterally because of the numerous reports indicating that branchial epithelia secrete Na^+ in seawater and resorb Na^+ in freshwater (*see* Maetz, 1971), and the directionality of a similar Na^+ transport mechanism in the operculum, if any, was unknown. The use of amiloride as a probe for transcellular Na^+ movements appears inappropriate, since it is effective only in "tight" epithelia. This tissue, however, is not strictly "leaky" in that it varies its resistance inversely with the external NaCl concentration, reaching well into the resistance range ($>800\ \Omega \cdot cm^2$) of "tight" epithelia at low external NaCl concentrations[2]. Also, in intact *F. heteroclitus*, the passive ionic permeability decreases 90% with adaptation to freshwater (Potts & Evans, 1967), and amiloride almost completely inhibits Na^+ influx in the freshwater adapted trout (Kirschner, Greenwald & Kerstetter, 1973). It appeared then, that the opercular epithelium was not strictly "leaky", could vary its ionic permeability *in vitro*, similar to the intact fish, and could possibly exhibit an amiloride sensitivity. Additional evidence against transcellular Na^+ movements is the lack of a direct effect of ouabain on the unidirectional Na^+ fluxes across the operculum (Degnan et al., 1977; Degnan & Zadunaisky, 1979).

The effects of TAP on the opercular epithelium were obviously not specific for a cation shunt, as evidenced by its inhibition of the active Cl^- efflux and the fact that the G_{Na^+}/G_T ratio remained unchanged. Possible amiloride-like effects of TAP on a transcellular Na^+ pathway across the operculum seem unlikely, since amiloride was without effect, both amiloride and TAP may possibly act at the same site (Balaban et al., 1979), and the K_I of amiloride is 1000 times less than that of TAP (Benos, Mandel & Balaban, 1979). The inhibition of the active Cl^- efflux can be explained by either a reduction in the P_{Na^+} or P_{K^+}. In a proposed model for Cl^- secretion (Silva et al., 1977*a*, *b*), Cl^- entry into the cell across the serosal membrane is tightly coupled to Na^+ moving across the membrane in response to its electrochemical gradient. Na^+ is then recycled back to the serosal side by the basolaterally located ATPase, and Cl^- passively diffuses down an electrical gradient across the mucosal membrane. A reduction in the serosal P_{Na^+} would inhibit the Cl^- entry step, and a reduction in the P_{K^+}, similar to that observed in the gallbladder (Reuss & Grady, 1979), would reduce both the Cl^- entry and exit by depolarizing the cell. TAP could also inhibit the Cl^- efflux by reducing the P_{Cl^-} of the mucosal membrane.

The present investigations also confirm the passive nature of the Cl^- influx, indicate the absence of a Cl^-/Cl^- exchange diffusion, and suggest that this influx is extracellular. The effect of a variety of compounds, including TAP, on the transcellular Cl^- efflux while having no effect on the Cl^- influx (Degnan et al., 1977; Degnan & Zadunaisky, 1979), can be taken as evidence favoring an extracellular Cl^- influx. In the operculum, TAP had no effect on the

[2] *See* footnote 1, p. 178.

Cl^- influx, similar to the observations in the gallbladder (Moreno, 1975*a*), or the unidirectional urea fluxes, contrary to the observations with the gallbladder (Moreno, 1975*b*). In the gallbladder, it was proposed that urea traversed the cation shunt, in addition to other pathways, and the inhibitory effect of TAP on the P_{urea} was mediated through its effect on this shunt. It is conceivable that urea traverses the Na^+ pathway in the operculum and that TAP acts to shield anionic charges in this shunt, which greatly reduce the Na^+ fluxes with little or no effect on the urea fluxes. However, if Cl^- also traverses this pathway, an increase in the Cl^- influx would be expected, but was not observed. Urea could also traverse the transcellular polar route, but again this seems unlikely in view of the inhibitory effects of TAP on these pathways. The data are more consistent with a Na^+-specific paracellular shunt in parallel with extracellular Cl^- and urea pathways. The similarities between the Cl^- influx and urea fluxes suggest that these two compounds may traverse the same extracellular pathway, but further studies are required to confirm this suggestion. The Cl^- influx and urea fluxes could also traverse the "leak" pathway. Unfortunately, there is no way to distinguish between a "leak" and a TAP-insensitive paracellular pathway.

The Maintenance of the Ionic Conductance by Na^+

The changes in the G_T in response to mucosal and serosal Na^+ substitutions were distinctly different. Mucosal Na^+ substitution reduced the G_T 76.0%, primarily by reducing the P_{Na^+} and secondarily by reducing the permeability of another ion(s). Serosal Na^+ substitution reduced the G_T 57.9%, with no significant change in the P_{Na^+}. Previous investigations have shown that mucosal Na^+ substitution reduced the active $J_{sm}^{Cl^-}$ and G_T 73.2% and 75.9%, respectively, while serosal Na^+ substitution reduced the $J_{sm}^{Cl^-}$ and G_T 71.4% and 50.7%, respectively.[3] The effect of mucosal Na^+ substitution on the G_T can therefore be attributed mostly to a reduction in the P_{Na^+} of the shunt pathway and the P_{Cl^-} of the apical membrane; while the effect of serosal Na^+ substitution appears to be on the P_{Cl^-} of the serosal membrane. This latter effect is in agreement with the coupled NaCl entry step in the proposed model for Cl^- secretion (Silva et al., 1977*a*, *b*), while the mucosal Na^+ sensitivity is a most interesting observation, especially in the light of recent morphological studies on Cl^- secreting epithelia.

In an elegant series of morphological studies on teleost gill epithelia, Sardet, Pisam and Maetz (1979) identified two distinct junctional complexes: (i) multistranded tight junctions between adjacent respiratory cells and adjacent respiratory and chloride cells, typical of those found in "tight" epithelia (Claude & Goodenough, 1973); and (ii) single-stranded shallow junctions between adjacent chloride cells, typical of those found in "leaky" epithelia (Claude & Goodenough, 1973). The multi-stranded junctions were unchanged by adaptation to fresh and salt water, did not allow the passage of lanthanum, and were suggested to have high electrical resistances. The single-stranded junctions arise as a consequence of salt-water adaptation, allow the passage of lanthanum, and were suggested to have low electrical resistances. Ernst, Dodson and Karnaky (1978) observed similar multi-stranded junctions between adjacent pavement cells and adjacent pavement and chloride cells, and similar single or double-stranded junctions between adjacent chloride cells in the opercular epithelium of *F. heteroclitus*. It would not be unreasonable to assume that the shallow chloride-chloride cell junctions in the opercular epithelium respond to fresh and salt water adaptation, similar to that observed in the gill (Sardet et al., 1979). The low resistance pathway between adjacent chloride cells would then correspond to the paracellular Na^+ shunt pathway, since it is sensitive to the external Na^+ concentration (salinity). The high resistance pathway between adjacent chloride and pavement cells could possibly correspond to the Cl^- influx pathway, and maybe the urea pathway, since it is insensitive to the external Na^+ concentration (salinity).

Comparison of the Findings with the Operculum to Those with the Gill

The present findings with the opercular epithelium agree with those of Kirschner, Greenwald and Sanders (1974), who contend that the Na^+ fluxes across the seawater-adapted trout gill are passive diffusional fluxes. They do not agree with the reports of Na^+/Na^+ and Cl^-/Cl^- exchanges (Motais, Garcia-Romeu & Maetz, 1966), Na^+/K^+ exchanges (Maetz, 1969), or active Na^+ effluxes (Potts & Eddy, 1973) across the seawater-adapted flounder gill. The observation which led to postulating these exchanges was the "trans" effect of external Na^+, K^+ and Cl^- on the branchial Na^+ and Cl^- efflux rates, resembling typical saturation kinetics. However, similar observations have been made with the isolated opercular epithelium preparation (Degnan & Zadunaisky, 1980*a*, *b*), where such transepithelial exchanges are not operative.

The transfer of fish from seawater to freshwater is

[3] *See* footnote 1, p. 178.

accompanied by a rapid reduction in the branchial Na^+ and Cl^- efflux rates (Motais et al., 1966; Epstein, Maetz & deRenzis, 1973), followed by a delayed secondary reduction, believed to be under neurohumoral control (Pickford & Phillips, 1959; Mayer-Gostan & Hirano, 1976). In the opercular epithelium preparation, lowering the mucosal Na^+ and/or Cl^- concentration reduces the Cl^- secretion rate (Degnan & Zadunaisky, 1980*a*, *b*), and lowering the mucosal Na^+ concentration, shown in the present study, reduces the Na^+ efflux rate. These changes, apparently limited by the turnover rate of the mucosal solution in these chambers, are rapid enough to account for the fast branchial efflux changes observed in intact fish. External Na^+ and Cl^- appear necessary to maintain the P_{Cl^-} of the apical membrane, while external Na^+ appears necessary to maintain the paracellular Na^+ shunt pathway and the overall transepithelial resistance. Such permeability changes explain the acute branchial response of euryhaline fish to changing salinities (before the neurohumoral factors become operative), the reduction in the branchial permeability when fish are transferred from seawater to freshwater, and the "trans" effect of external Na^+ and Cl^- on the branchial efflux rates without invoking exchange diffusion.

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