

POTASSIUM INFLUX ACROSS FLOUNDER INTESTINAL BRUSH BORDER: Na AND Cl DEPENDENCE AND EFFECT OF OUABAIN

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Unidirectional Rb or K influx across the brush border of flounder intestinal epithelium is dependent on luminal Na and Cl and is inhibited by loop diuretics such as furosemide (Musch et al, *Nature* 300:351, 1982). Previous NaCl influx studies suggested a 1:1 NaCl stoichiometry (Frizzell et al, *J. Memb. Biol.* 46:27, 1979) and a 1:2 RbCl stoichiometry (Field et al, *Bull MDIBL* 21:93, 1981) for the diuretic-inhibitable influx. The present studies were designed to determine (1) the NaKCl stoichiometry of this transporter and (2) the effect of serosal ouabain on ^{86}Rb influx.

METHODS--Following a 30-60 min preincubation, the unidirectional influx of ^{86}Rb from mucosal medium into the epithelium (J_{me}^{Rb}) was measured under short-circuit conditions in modified Ussing-type chambers as previously described (Frizzell et al, *J. Memb. Biol.* 46:27, 1979). The standard Ringer contained in mmol/L: NaCl 160, RbCl 5, CaCl_2 1.25, MgCl_2 1.1, NaH_2PO_4 0.3, Na_2HPO_4 1.65 and D-glucose 20 and was buffered to pH 8.0 with 5 mM N-2-hydroxyethyl piperazine propane sulfonic acid (EPPS). Solutions were bubbled with room air and maintained at 15°C. In ion replacement studies Na was replaced with either tetraethylammonium (TEA) or N-methyl-D-glucamine (NMDG); Cl was replaced with gluconate (Na, Ca, and Mg salts) and NO_3^- (Rb salt) and to determine Rb dependence, the [NaCl] was varied reciprocally with RbCl.

RESULTS--Na, Cl, and Rb Dependence of ^{86}Rb Influx. The Rb concentration dependence of ^{86}Rb influx is shown in Figure 1. The Hill coefficient for Rb-dependence of 1.19. Na and Cl concentration dependences of Rb influx are shown in Figure 2. The influx remaining in the absence of Cl or Na is assumed to have been paracellular.

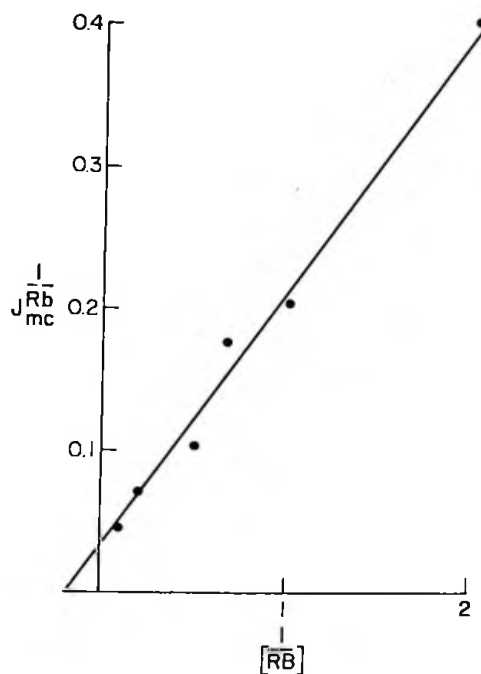


Figure 1.--Lineweaver-Burk plot of the Rb-dependence of ^{86}Rb influx. $K_t = 6 \text{ mM}$.

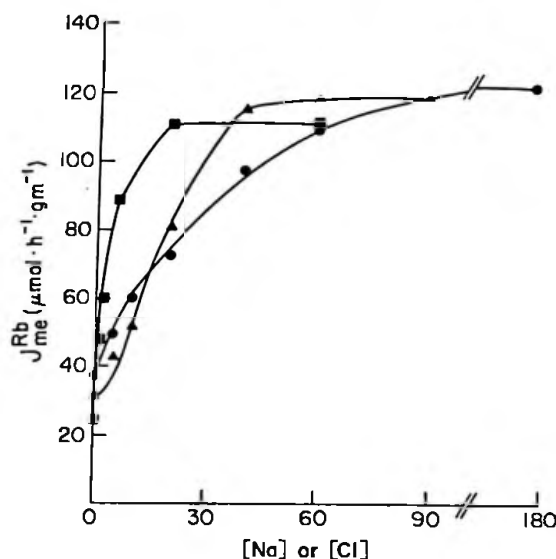


Figure 2.-- ^{86}Rb influxes as functions of medium Cl concentration (Δ , gluconate replacement) and medium Na concentration (\square , NMDG replacement and \circ , TEA replacement).

The Na-dependence curve, when Na is replaced by NMDG obeys simple Michaelis-Menten kinetics and has a Hill coefficient of 1.09. Neither the Cl dependence nor the Na-dependence when Na was replaced by TEA follow simple Michaelis-Menten kinetics, however, the Hill coefficient for Cl-dependence is 2.06 and the Hill plot for Na-dependence (TEA substitution) is biphasic ($K_{h1}=0.2$; $K_{h2}=2.28$). Tetramethylammonium and choline were also tested as substitutes for Na but both inhibited Rb influx: with either, the Rb influx at 60 mM Na was less than twice that at 0mM Na.

Simultaneous influxes into the same tissues of (^{22}Na and ^{42}K) or (^{36}Cl and ^{42}K) were also measured (Table 1).

TABLE 1. SIMULTANEOUSLY MEASURED INFLUXES OF ^{42}K AND ^{22}Na OR ^{42}K AND ^{36}Cl

	J_{me}^{Na}	J_{me}^{K}	J_{me}^{Cl}	J_{me}^{K}
Control	204 \pm 33	62 \pm 9	182 \pm 33	181 \pm 9
Furosemide (0.4mM)	118 \pm 27	33 \pm 4	74 \pm 21	136 \pm 10

Δ	86	28	108	45
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Means \pm 1 SE for 4 paired experiments in each set (i.e., Na&K and Cl&K). Units are $\mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{gm wet wt}^{-1}$. Na and K influxes were measured at 60mM Na and 180mM Cl concentrations and Cl and K influxes were measured at 60mM Cl and 180mM Na concentrations. [K] was 5mM in both cases.

Effect of Ouabain--We also determined the effect on Rb influx of ouabain (0.1 mM serosal side addition), which blocks the Na/K pump and also Cl transport in this tissue. Rb influxes were measured after the Isc had decreased to nearly zero (usually 45-60 min). These experiments were performed in both the presence and absence of serosal calcium (0.2mM EGTA added) since ouabain may indirectly increase cellular [Ca] and thereby inhibit salt absorption (see Donowitz et al., Bull. MDIBL 21:24, 1981). Ouabain greatly reduced ^{86}Rb influx, but this effect was not prevented by omitting serosal Ca (Table 2). Because cGMP inhibits Na, K, Cl cotransport in flounder intestine (see Rao et al,

TABLE 2. EFFECTS ON RB INFLUX OF OUABAIN AND/OR REMOVAL OF CA FROM THE SEROSAL MEDIUM.

	J_{me}^{Rb}	Isc	G_t
+Ca _s (control)	135.8 \pm 9.8	-2.89 \pm 0.36	30.8 \pm 2.0
+Ca _s + ouabain	38.8 \pm 7.7 **	0.86 \pm 0.27 **	27.2 \pm 1.0
-Ca _s	90.3 \pm 11.2	-1.97 \pm 0.61	42.0 \pm 3.3 *
-Ca _s + ouabain	32.8 \pm 12.1 **	-0.01 \pm 0.11 **	35.2 \pm 6.4

Values are means \pm 1 SE for 4 paired experiments. Units for each are; J_{me} $\mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{gm wet wt}^{-1}$, Isc $\mu\text{Eq}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$, and G_t mS $\cdot\text{cm}^{-2}$. Ca was removed from the serosal side only 75-90 min before influx measurements. *p<0.05 different from control, **p<0.01 different from control.

this bulletin), we measured cGMP concentration in the mucosa 45-60 min following ouabain addition. Ouabain increased mucosal [cGMP] 5-fold, from 0.24 \pm 0.13 to 1.18 \pm 0.54 (3 experiments).

DISCUSSION--Both the slopes of their Hill plots and simultaneous ^{36}Cl and ^{42}K influxes support the hypothesis of a Na-K-Cl cotransporter which requires 1 K and 2 Cl. The number of Na sites is uncertain, however, and the Na requirement possibly varies with the cation used to replace Na. When NMDG is used, it appears that only one Na is required (slope of the Hill plot = 1.09). The simultaneous ^{22}Na and ^{42}K influxes suggest that 3 Na may be trans-

ported per K. In these experiments, however, TEA was substituted for Na and the Hill plot for Rb influx when Na is replaced by TEA is biphasic. Although at 60 mM Na (the concentration used for the simultaneous ^{22}Na and ^{42}K influxes) the slope of the Hill plot is close to 2.0, the non-linearity renders any estimate of stoichiometry tenuous. Possibly there are more than one brush border cotransport systems for NaCl with varying K requirements and varying Na:Cl stoichiometries.

The effect of ouabain indicates that there is some type of feedback between the two borders of the cell to control electrolyte transport. The fact that ouabain increases cellular cGMP 5-fold suggests that this cyclic nucleotide may mediate the feedback inhibition and reinforces its central role in regulating intestinal salt absorption in the flounder.

MEASUREMENTS OF MEMBRANE POTENTIAL AND MEMBRANE RESISTANCE IN ISOLATED CELLS FROM DOGFISH RECTAL GLANDS

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The active secretion of Cl^- in the rectal gland of the dogfish (*Squalus acanthias*) is enhanced by c-AMP or theophylline (a phosphodiesterase inhibitor). Cl^- is thought to be actively transported across the baso-lateral membrane and to be passively extruded across the luminal membrane into the duct (Silva et al., Am. J. Physiol. 233:F298-F306, 1977). According to this scheme, Cl^- secretion would be enhanced by either hyperpolarization or increased Cl^- conductance. The suppression of the Cl^- secretion in the isolated perfused gland by Ba^{2+} is consistent with this theory since Ba^{2+} is expected to depolarize the gland by blocking K^+ channels (Silva et al., MDIBL Bull 21:12-13, 1981). To study the electrophysiology of the secretory process we decided to use the enzymatically separated single cell preparation (Segall et al., MDIBL Bull. 20:38-39, 1980) to measure changes in membrane potential and membrane conductance which might occur following activation by c-AMP. Judged by changes in O_2 consumption, single cells are known to respond much like the intact gland to c-AMP, furosemide, ouabain and changes in the Na^+ and Cl^- concentrations (Spokes et al., MDIBL Bull. 21:13-14, 1981).

METHOD--Single cells were suspended in Shark-Ringer (in millimoles per liter: Na, 280; K, 5; Cl, 280; bicarbonate, 8; Ca, 5.0; Mg 3.0; phosphate, 1; sulphate, 0.5; urea 350; glucose, 20) and were impaled by single conventional microelectrodes (3M KCl, 5-30 Mohm) on the stage of an inverted microscope.

RESULTS--Seen under the microscope the cellular suspension consisted of spherical single cells, clusters of a few (2-10) spherical cells and some larger tubular fragments. Impalement of single cells or cells in small clusters yielded membrane potentials close to -100 mV. Strict screening of the results was performed to verify this surprisingly large membrane potential. Results were considered reliable only if the potential dropped abruptly upon impalement and was stable for several minutes. In some cases the measured membrane potential hyperpolarized slowly by 10 to 20 mV following impalement. In these cases the electrode was often pulled out to check if the tip potential had changed. Taking such precautions, the resting membrane potential was found to be $-90 \text{ mV} \pm 12 \text{ mV}$ (S.D., n=45).

In order to measure the membrane conductance of the single cell preparations current pulses of varying amplitude were injected through the intracellular electrode and the resulting potential deflection was measured with the same electrode (Figure 1a). This method depends critically on the proper compensation for stray capacitance and electrode resistance. The compensation was carried out always before impalement. Figure 1a shows that the voltage transients resulting from uncompensated capacitance are substantially faster than the charging of the membrane capacitance. The records suggest a small change in the electrode resistance following impalement. Readjustment of the resistance compensation would improve the appearance of the records without actually improving the accuracy