Biochimica et Biophysica Acta, 685 (1982) 383–385
Elsevier Biomedical Press

BBA Report

BBA 70017

OUABAIN-SENSITIVE $^{86}Rb(K)$ INFLUX IS LINKED TO TRANSEPITHELIAL Na TRANSPORT IN PIG KIDNEY CELL LINE

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(Received August 24th, 1981)
(Revised manuscript received December 15th, 1981)

Key words: Rb^+ influx; $(Na^+ + K^+)$-ATPase; Na^+ transport; Co-transport; Ouabain sensitivity; (Pig kidney)

The pig kidney cell line, LLC-PK_1, exhibits rheogenic D-glucose coupled transepithelial Na^+ transport that is inhibited by phlorizin. By measuring the difference in initial rates of influx of $^{86}Rb^+$ with and without coupled Na^+ transport, we can demonstrate an $^{86}Rb^+$ uptake linked to Na^+ transport. The simultaneous determination of phlorizin-inhibited Na coupled D-[^{3}H]glucose uptake and $^{86}Rb^+$ influx allows calculation of an Na^+/Rb^+ stoichiometry that is consistent with an electrogenic Na^+ for Rb^+ exchange.

Present understanding of transepithelial sodium transport is based on the two-membrane model originally proposed for frog skin by Koefoed-Johnson and Ussing [1]. According to the model, Na^+ enters epithelial cells across the apical membrane and then is pumped out at the opposite basolateral membrane in exchange for potassium. This model requires a coupling between basolateral K^+ influx and transepithelial Na transport; yet despite attempts to confirm this aspect of the model, there has been no compelling evidence for or against it.

We used cell culture techniques to form a simplified epithelial tissue a homogeneous monolayer of polarized transporting epithelial cells. The pig kidney cell line LLC-PK_1 exhibits Na^+-dependent D-glucose coupled transport [2–4] and when cultured on a membrane filter and placed in Ussing chamber, the net apical-to-basolateral $^{22}Na^+$ flux is equivalent to the short circuit current, I_{sc}, across the cell layer. Net Na^+ transport occurs only as a function of Na^+-D-glucose coupled transport [2]. Since apical Na^+ and D-glucose entry into the cells are coupled, we have used D-[^{3}H]glucose uptake as a marker for Na^+ entry. $^{86}Rb^+$ can substitute for K^+ in $(Na^+ + K^+)$-ATPase function and was used to indicate K^+ influx [5]. We disrupted confluent monolayers and determined simultaneous initial rates of uptake of D-[^{3}H]glucose and $^{86}Rb^+$ with and without phlorizin. Any difference in upake under these two conditions is presumably due to the function of the coupled Na^+, D-glucose transport pathway.

LLC-PK_1 cells passages 184–190, split weekly at 1:3 were grown and mainained at confluence (7–9 days) in 35 mm dishes under standard tissue culture conditions *. Before use, the growth medium was removed and replaced with Hanks' balanced salt solution ** with and without phlorizin (10^{-4} M). The cell layer was loosened from the

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

* Culture conditions: cells were grown in a 1:1 mixture of Eagle's minimum essential medium and Hams F12 (GIBCO) plus 10% calf serum, 5 μg/ml insulin and 10 μg/ml gentamycin at 37°C in 5% CO_2/95% air.

** Composition of Hanks' balanced salt solution: 140 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.36 mM Na_2HPO_4, 5.5 mM D-glucose, 10 mM Hepes buffered at pH 7.2.

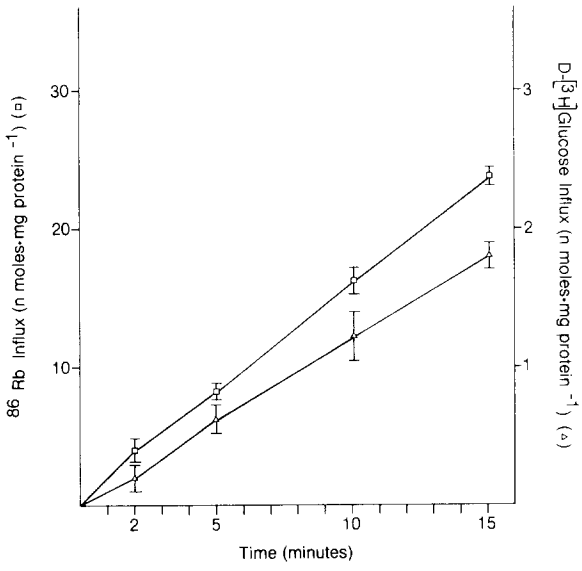

Fig. 1. Initial rates of D-[^{3}H]glucose (△) and ^{86}Rb (□) uptake into loosened cell culture monolayers of LLC-PK$_1$.

dish with a rubber policeman to provide solution access to the basolateral surface. Isotope solution was added to dishes and ten minutes later the cells were poured over an 8 μm membrane filter (25 mm Millipore, type SCWP) maintained under suction. The culture dish and filter were washed with a total of 60 ml ice-cold phosphate buffered saline and the filter was placed in a liquid scintillation vial with 8 ml of Biofluor (New England Nuclear) and counted in a dual channel counter. Specific activity for Rb^+ fluxes was calculated from cpm $^{86}Rb^+/[K^+]$. Separate experiment determined that the cellular uptake of D-[^{3}H]glucose and $^{86}Rb^+$ was linear over 15 min (Fig. 1). In experiments with glucose-free Hanks' balanced salt solution, intact monolayers were incubated for 12 h in glucose-free Hanks' balanced salt solution and fresh solution was used before beginning the experiment. In the ouabain experiments cells were pretreated with ouabain (10^{-4} M) for 5 min after loosening prior to isotope addition. All experiments were conducted at 37°C. Protein determinations were made on similarly disrupted monolayers trapped on membrane filters by the Biorad protein assay [6].

Our results (Table I) indicate that when Na^+-coupled D-glucose uptake is inhibited by phlorizin (10^{-4} M) both D-[^{3}H]glucose and $^{86}Rb^+$ influx are reduced. Phlorizin is specific only for the coupled apical Na^+, D-glucose uptake and not for Rb^+ influx. This is shown by the lack of a phlorizin effect on $^{86}Rb^+$ influx in the absence of D-glucose. The addition of ouabain (10^{-4} M) inhibited $^{86}Rb^+$ entry suggesting that the $^{86}Rb^+$ influx occurs via the $(Na^+ + K^+)$-ATPase.

We do not believe the decreased Rb^+ uptake in the presence of phlorizin is because of reduced D-glucose uptake and availability as a metabolic substrate. If Rb^+ uptake were linked to D-glucose metabolism and ATP synthesis the stoichiometry

TABLE I

INITIAL RATES OF INFLUX OF D-[^{3}H]GLUCOSE AND ^{86}Rb INTO DISRUPTED LLC-PK$_1$ MONOLAYERS

HBSS, Hanks' balanced salt solution.

Conditions	Influx (nmol·min^{-1}·mg protein^{-1})			
	D-[^{3}H]Glucose		^{86}Rb	
1 Control (HBSS)	4.07 ± 0.214 (18) [a]		13.8 ± 0.52 (18)	
+ phlorizin (10^{-4} M)	2.56 ± 0.10 (18)		11.7 ± 0.38 (18)	
Δ(control − phlorizin)	1.51 ± 0.26	$P<0.05$ [b]	2.18 ± 0.64	$P<0.05$
2 Glucose-free HBSS	–		7.69 ± 0.36 (8)	$P>0.05$
+ phlorizin (10^{-4} M)	–		7.51 ± 0.39 (8)	
3 HBSS + ouabain (10^{-4} M)	2.76 ± 0.12 (12)	$P>0.05$	2.29 ± 0.14 (12)	$P>0.05$
+ phlorizin (10^{-4} M)	2.60 ± 0.17 (12)		2.34 ± 0.09 (12)	

[a] $\bar{x} \pm$ S.E. (n).

[b] Student's t-test.

would be far in excess of the 1.5 : 2.2, D-glucose : Rb^+ of these studies. The effect of phlorizin on D-glucose uptake although completely able to inhibit coupled uptake [2] reduces total uptake only 37% with significant Na^+-independent uptake remaining.

These results indicate a coupling between transepithelial Na^+ transport and Rb^+ uptake. As a uniform population of transporting epithelial cells, the LLC-PK_1 monolayer provides the smallest possible cellular compartment for detection of Rb^+ influx linked to coupled Na transport. Even under these conditions the Na^+ transport specific Rb^+ influx was only 15% of the total which may explain the inability to show this linkage in more complex epithelial tissues [7–10].

From the relation of D-[^{3}H]glucose to $^{86}Rb^+$ entry (Table I) and the additional finding from our laboratory that two sodium ions are required for each molecule of glucose that enters the cell via the co-transport system [11,12], we can calculate an Na^+/Rb^+ ratio (R) for these cells:

$$R = 2\left(J^{in}_{\text{glucose}} - J^{in}_{\text{glucose+Pz}}\right) / \left(J^{in}_{\text{Rb}} - J^{in}_{\text{Rb+Pz}}\right)$$

where influx, J^{in}, is the mean determination for each experimental condition; i.e., with and without phlorizin (Pz). Since each mean has an associated variance, an estimate of the ratio, R, and its confidence interval was calculated using the 'jackknife method' [13]. From this analysis R is 1.2 ± 0.32 ($\bar{x} \pm$ S.D.). This ratio is consistent with the mechanism of Na^+ for K^+ exchange via the ($Na^+ + K^+$)-ATPase and a coupling stoichiometry of 3 Na^+ to 2 K^+ as reported for other epithelia [14–16].

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