LETTER TO THE EDITOR

Na/Na Exchange Through the Na/K Pump of HK Sheep Erythrocytes

Dear Sir:

In their classic paper of 1960, Tosteson and Hoffman (13) provided the physiological basis for the HK/LK polymorphism of sheep erythrocytes (see Lauf [11] for a recent review). In their results, the function of the Na/K pump in HK sheep cells differed in one important respect from pumps in human red cells. Garrahan and Glynn (5) showed that human red cell pumps, in the absence of extracellular K, exchange Na for Na, 1:1. Tosteson and Hoffman (13) reported that there was no Na/Na exchange through the pumps in normal HK sheep cells. They did report a substantial Na/Na exchange independent of the pumps, i.e., Na/Na exchange insensitive to ouabain, similar to a mode of Na/Na exchange observed more recently in human red cells (12).

If Na/K pumps differ in this respect between sheep and human red cells, there may be important implications for our understanding of the mechanism of the pump, much of which is based upon the demonstration and characterization of various modes of its operation in human red cells (7). ATP/ADP exchange, first demonstrated in Na,K-ATPase from eel electric organ (4), and characterized more recently in human red cell ghosts (8), can be understood in terms of its relationship to Na/Na exchange (7, 9). HK sheep red cell membranes catalyze ouabain-sensitive ATP/ADP exchange (14); if HK red cell Na/K pumps cannot mediate Na/Na exchange, then the significance of ATP/ADP exchange should be reconsidered.

We have re-examined the function of the Na/K pump of HK sheep red cells in the hope of determining if Na/Na exchange through it is possible. Mindful that both ADP and ATP are required for Na/Na exchange in human cells (6), and that nucleotide concentrations are lower in sheep than in human cells (1), we incubated the cells with appropriate metabolic substrates in order to increase the nucleotide concentrations. We also raised intracellular Na concentrations by treatment with nystatin. We were able to demonstrate Na/Na exchange through the pumps of the HK sheep cells.

Blood was drawn into heparin from the jugular vein of adult Suffolk HK sheep. The cells were then washed twice by centrifugation and resuspension in an isotonic medium containing (mM): 150 NaCl, 10 Tris-HCl, 5 glucose, pH 7.4. To raise the intracellular nucleotide concentrations, aliquots of cells were incubated for 1 h at 37°C, 5% hematocrit, in a medium containing (mM): 150 NaCl,

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10 adenosine, 5 glucose, 10 Tris- H_3PO_4 , pH 7.4. Control suspensions were incubated at the same time in (mM): 150 NaCl, 10 sucrose, 10 Tris-HCl, pH 7.4. To modify intracellular Na concentrations, cells were treated with nystatin as described previously (2). To load the cells with ^{22}Na (for Na effluxes), the tracer was included during the last 20 min of the incubation with nystatin. After washing them free of nystatin, the cells were incubated for an additional 30 min in either the adenosine-phosphate medium or the sucrose medium described above. Then the cells were washed twice by centrifugation in an isotonic medium with choline substituted for Na.

Na effluxes were measured and calculated as described before (3). For all of the aliquots of cells, the efflux media contained (mM): 160 NaCl or choline Cl, 10 Tris-HCl, pH 7.4. Some of the media contained ouabain (0.1 mM). The fluxes were measured in duplicate flasks at 37°C, ~2% hematocrit, with samples taken at 0, 20, and 40 min. Na influxes were measured by a modification of the method described before (3). The media for the influxes were the K-free adenosine-phosphate or sucrose media described above. Some of the media contained ouabain (0.1 mM). Intracellular Na concentrations, $[\text{Na}]_i$, were obtained from flame photometric analyses of lysates of suspensions of cells washed in Na-free medium and the hematocrits of the suspensions.

Intracellular ATP and ADP were measured as described before (8, 10) on supernatant solutions of boiled cell suspensions taken either just before or while measuring the fluxes. Concentrations were calculated using the hematocrits of the suspensions.

Table I shows Na effluxes from cells that had been preincubated in media either with or without adenosine plus orthophosphate. The nucleotide concentrations for the cells in this experiment and in the experiments in Table II will be presented in Table III. The effluxes were measured in four types of media: (a) with Na; (b) with Na plus ouabain; (c) Na-free (choline the substitute cation); and (d) with choline plus ouabain. Fluxes through the pump are given by the differences between fluxes measured with and without ouabain. Na/Na exchange through the pump, given by the ouabain-inhibitable fluxes in Na, was measurable in both types of cells, although it was threefold larger in adenosine-phosphate cells. There was measurable uncoupled Na efflux through the pump (the ouabain-inhibitable flux into choline medium) in adenosine-phosphate cells. In designating ouabain-inhibitable Na efflux into Na medium as Na/Na exchange, and into choline medium as uncoupled Na efflux, it is assumed that external Na does not stimulate the uncoupled efflux. Table I also shows a large component of ouabain-insensitive Na/Na exchange; it was the same in adenosine-phosphate and sucrose cells.

It is possible that some of ouabain-inhibitable Na efflux in nominally K-free medium is activated by K that had leaked from the cells, rather than by external Na, even though replacing Na with choline reduced the flux. That is to say, some of Na efflux ascribed to Na/Na exchange may have been Na/K exchange, i.e., the pump operating in its normal mode. Therefore, we measured ouabain-inhibitable Na influxes, both in cells that had been preincubated in the adenosine-phosphate medium and in cells preincubated in sucrose medium. The results of

three influx experiments are shown in Table II. In the first, $[\text{Na}]_c$ was varied between 20 and 90 mmol/liter cells. Experiments 2 and 3 were carried out only with high $[\text{Na}]_c$ cells. In addition, ouabain-inhibitable Na efflux was measured simultaneously with influx in experiment 3. The results of these three experiments show that there was a ouabain-inhibitable Na influx in adenosine-phosphate cells at $[\text{Na}]_c$ of 60 mmol/liter and higher, but probably not at lower Na concentrations. In experiment 1, there was no significant ouabain-inhibitable Na/Na exchange in the sucrose cells at any $[\text{Na}]_c$. In experiments 2 and 3, there was Na/Na exchange in sucrose cells, although at a lower rate than in adenosine-phosphate cells. The highest ouabain-inhibitable Na fluxes in Table I (efflux) and Table II (influx and efflux) were similar, which is consistent with 1:1 Na/

TABLE I
Na Efflux from HK Sheep Erythrocytes

Flux medium (mM)	Na efflux	
	Adenosine-P _i cells	Sucrose cells
	mmol/liter · h	
NaCl (160)	2.97±0.04	2.66±0.06
+ ouabain	2.19±0.03	2.42±0.01
Choline Cl (160)	0.86±0.02	0.79*
+ ouabain	0.60±0.09	0.71±0.04
Summary of fluxes		
Na/Na exchange, ouabain-inhibitable	0.78	0.24
Uncoupled Na efflux, ouabain-inhibitable	0.26	0.09
Na/Na exchange, ouabain-insensitive	1.60	1.71

Aliquots of the cells had been treated so as to raise their nucleotide concentrations (adenosine-P_i cells). Na concentrations, $[\text{Na}]_c$, had been raised in all of the cells to 73.4 ± 1.2 mmol/liter cells (SD, $n = 8$). Nucleotide concentrations in the two types of cells are given in Table III. Compositions of the media (all K-free) are given in the text. Fluxes are expressed as mmol/liter cells · h $\pm$ total range ($n = 2$). Similar results were obtained in two other experiments.

* Single determination.

Na exchange. The similarity of the simultaneously measured efflux and influx in experiment 3, Table II, strongly supports the 1:1 stoichiometry. The Na influx inhibited by ouabain is unlikely to be by any mode of the pump other than Na/Na exchange. It was promoted by intracellular Na, and the only other possible mode, complete reversal of the pump, probably would have been inhibited by high $[\text{Na}]_c$.

Table III shows the ATP and ADP concentrations in the cells in the experiments in Tables I and II. In every experiment, cells incubated with adenosine-phosphate contained higher concentrations of both nucleotides than did cells incubated without the substrates (sucrose cells). The relative reductions of ATP and ADP varied among the experiments; the sums of the two for adenosine cells and sucrose cells varied less. The mean ratio of sums of nucleotide concentrations,

adenosine cells to sucrose cells, is 2.36 ± 0.47 for the four experiments in Table III. The corresponding mean ratio for Na/Na exchange, adenosine cells to sucrose cells, is 2.80 ± 0.66 for the same four experiments. Therefore, Na/Na exchange and the nucleotide concentrations are correlated.

Our results provide a clear demonstration that adenine nucleotides promote ouabain-sensitive Na fluxes in HK sheep red cells in K-free media. (No attempt

TABLE II
Ouabain-inhibitable Na Influx in HK Sheep Erythrocytes

[Na] _e <i>mmol/liter cells</i>	Na influx, ouabain-inhibitable <i>mmol/liter · h</i>	
	Adenosine-P _i cells	Sucrose cells
Experiment 1		
20	0.09±0.10	-0.09±0.22
33	0.24±0.17	-0.06±0.19
60	0.45±0.19	0.21±0.12
92	0.93±0.27	0.21±0.19
Experiment 2		
84	0.81±0.24	0.40±0.10
Experiment 3		
81	0.78±0.08	0.41±0.24
	Na efflux	
81	0.70±0.06	0.59±0.08

Results of three experiments are shown. In experiment 3, efflux was measured simultaneously with influx. Aliquots of cells had been treated so as to raise their nucleotide concentrations (adenosine-P_i cells). [Na]_e was modified as indicated in the left column, and was the same (within 4%) at each level of [Na]_e for adenosine-P_i and sucrose cells. Cell nucleotide concentrations from these experiments are shown in Table III. The influxes were measured in triplicate in the K-free media described in the text. The efflux (experiment 3) was measured as in Table I, but in 150 mM Na media. Both types of flux are expressed in mmol/liter · h. Shown are the differences between total fluxes and fluxes in the presence of ouabain. Errors for influxes are standard deviations of differences, $\sqrt{SD_1^2 + SD_2^2}$, where the subscripts denote SD's of the two fluxes. Errors of effluxes are total ranges of two estimates of ouabain-inhibitable efflux after subtracting a single (mean) value for ouabain-insensitive efflux. The total range for estimates of the latter did not exceed 0.15 mmol/liter · h. In experiment 1, ouabain-insensitive Na influx was 1.97 mmol/liter · h in cells with 20 mmol/liter Na and increased to 2.31 in cells with 92 mmol/liter Na. It was the same (within 12%) in adenosine-P_i and sucrose cells.

was made to determine the relative affinities of ATP and ADP for the pump.) The evidence that these fluxes are Na/Na exchange comes from measurements of both influx and efflux of Na: that influx of Na is promoted by intracellular Na, and efflux by external Na. The results of either type of experiment are subject to other interpretations; together they provide strong support for our conclusion of Na/Na exchange through the pump. Whittington and Blostein (14) had shown ATP/ADP exchange in permeable ghosts of HK sheep red cells

using a Na concentration of 50 mM. With the correlation of Na/Na and ATP/ADP exchanges by the pump in human red cells (5, 6, 8, 9), it is reasonable to suppose that Na/Na and ATP/ADP exchanges are reflections of the same activity of the pump in sheep cells as well.

Tosteson and Hoffman (13) concluded that there was no Na/Na exchange mediated by the pump in HK sheep red cells. They had not attempted to raise either Na or nucleotide concentrations in the cells. (It is worth noting that in Table VI of their paper [13], there appears to be a small strophanthidin-

TABLE III
Nucleotide Concentrations in HK Sheep Erythrocytes Incubated with Metabolites (Adenosine plus Orthophosphate Cells) or without Metabolites (Sucrose Cells)

Cells	Nucleotides		
	ATP	ADP	Σ
	<i>mmol/liter cells</i>		
(Table I)			
Adenosine + P_i	0.48 $\pm$ 0.03	1.10 $\pm$ 0.03	1.58
Sucrose	0.39 $\pm$ 0.01	0.57 $\pm$ 0.02	0.96
(Table II, experiment 1)			
Adenosine + P_i	0.63 $\pm$ 0.06	1.67 $\pm$ 0.28	2.30
Sucrose	0.038 $\pm$ 0.002	0.58 $\pm$ 0.09	0.62
(Table II, experiment 2)			
Adenosine + P_i	0.96 $\pm$ 0.02	1.01 $\pm$ 0.09	1.97
Sucrose	0.13 $\pm$ 0.001	0.75 $\pm$ 0.02	0.88
(Table II, experiment 3)			
Adenosine + P_i	0.69 $\pm$ 0.02	1.30 $\pm$ 0.02	1.99
Sucrose	0.15 $\pm$ 0.004	0.94 $\pm$ 0.03	1.09

Analyses are shown for cells from flux experiments in Tables I and II. ATP and ADP were extracted and measured as described in the text. Shown are means $\pm$ SD ($n = 3$) for replicate determinations on one sample of extract, except for experiment 1, Table II, where means are of single determinations on separate extracts ($n = 4$). Σ represents sums of the ATP and ADP concentrations. Their concentrations in fresh sheep red cells are 1.0 ATP and 0.2 ADP (mmol/liter cells; reference 1).

inhibitable Na efflux from HK cells in K-free medium, although it is not mentioned in the text.) Now, with the demonstration of Na/Na exchange mediated by the Na/K pump in HK sheep erythrocytes, there is no reason to suppose that the mechanisms of Na-dependent ATP/ADP exchange and the associated Na transport differ in human and sheep red cells.

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