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Ouabain-Insensitive Transintestinal Transport in the Rat Jejunum Incubated *in Vitro* (42716)

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Abstract. The jejunal tract of rat intestine, everted and incubated *in vitro* at 28°C for 2 hr in Krebs–Ringer bicarbonate solution, was used to test the existence of a ouabain-insensitive sodium pump. Cell water, Na, and K together with Na, fluid, K, and lactate transported into the serosal compartment were determined and, under control conditions, the tested parameters were found constant in time. By blocking the Na–K pump with 20 m*M* ouabain in the serosal compartment, the enterocyte lost K and gained Na, but the cell volume did not vary. Moreover, the transport of Na, fluid, and lactate, although lower, was constant for 2 hr. When ethacrynate was added or when the ATP supply was blocked by adding 2,4-dinitrophenol plus iodoacetate, the cell swelled and the transport of Na and fluid stopped. These results are interpreted as suggesting the existence of a ouabain-insensitive Na pump, in addition to the well-known Na–K pump.

Everted intestines incubated *in vitro* have been used in the last decades to study the transintestinal transport of fluid, electrolytes, and nutrients. The everted condition allows easy entrance of the substances at the mucosal pole and, after crossing the tissue (considered to be a "black box"), they can be detected in the serosal fluid.

Epithelial cells are specialized to promote transcellular transport of Na by means of an asymmetric localization of the Na–K pump (basolateral membrane). Evidence exists that there is another pump, which actively transports only Na out of the cell, in addition to the known Na–K pump. This second Na pump, energized by a ouabain-insensitive Na–ATPase, has been characterized enzymatically in basolateral membranes of guinea pig kidney (1, 2), rat kidney (3), and guinea pig small intestine (4). Uptake studies in basolateral membrane vesicles from rat kidney (5, 6) and guinea pig small intestine (7) have also demonstrated this Na–ATPase activity. It has been suggested that its role is the regulation of cellular volume and that its activity is modulated by micromolar concentrations of calcium (6).

The aim of the present study was to investigate the presence of such a mechanism in an intact tissue, by using the everted sac preparation of rat jejunum. Our results are consistent with the existence of a second Na pump, in addition to the well-known Na–K pump, which functions in the basolateral membrane of the enterocyte.

Materials and Methods. The rat jejunum, everted and cannulated, was incubated at 28°C in 50 ml Krebs–Ringer bicarbonate solution gassed with 95% O_2 and 5% CO_2 and with added 5.56 m*M* glucose and 0.5 m*M* phenol red (or, in a few experiments, trace amounts of [^{14}C]polyethylene glycol (PEG, mol wt 4000)). Three milliliters of the same solution was placed in the serosal compartment and net transintestinal fluid transport was determined every 20 min from the phenol red dilution. As in a previous study from this laboratory (8) the mucosal fluid was vigorously bubbled to avoid the formation of unstirred layers. In a few experiments the serosal solution was also recirculated by a peristaltic pump at about 5 ml/min and vigorously bubbled, as in (8), but this last maneuver did not change the results. In some cases phenol red (or PEG 4000) was added only to the serosal solution in order to evaluate the serosal extracellular space. Na, K (flame photometry, I.L. Model 943), and lactate (kit, Boehringer–Mannheim, West Germany) were also determined. Two groups of experiments with or without (=control) 20 m*M* ouabain in the serosal fluid were performed. In two other groups of experiments, in addition to ouabain in the

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serosal compartment, both incubation fluids had added 2 m*M* ethacrynate or 0.1 m*M* 2, 4-dinitrophenol (DNP) plus 3 m*M* iodoacetate (MIA). After 10 min preincubation, experiments lasting 60 or 120 min were performed. Cell Na, K, and water were determined in the mucosal layer, scraped off either at the end of the experiment or just after the preincubation period. Intracellular Na and K concentrations were obtained after correction for the extracellular spaces (marker phenol red or PEG). Details and controls of this technique have already been published (9). For statistical analysis Student's *t* test for unpaired data was used.

Results. Results are presented in Table I and Figs. 1–4. Table I shows that under control conditions the wet weight/dry weight ratio of the scraped mucosa did not change after 2 hr incubation of the everted intestine at 28°C; a similar pattern was observed for cell water, Na, and K; i.e., none of these parameters varied significantly during 2 hr incubation. The addition of 20 m*M* ouabain to the serosal fluid did not cause a statistically significant variation in the wet weight/dry weight ratio or in cell water with respect to control values, even after 2 hr incubation. The pattern of Na and K was different: the cell gained the former and lost the latter during both preincubation and incubation periods. When in addition to serosal ouabain, the incubation fluids included either 2 m*M* ethacrynate or 0.1 m*M* DNP plus 3 m*M* MIA, the wet weight/dry weight ratio rose abnormally both after 1 hr (6.9 ± 0.4 and 8.2 ± 0.4, respectively) and after 2 hr (7.5 ± 0.3 and 9.7 ± 0.5, respectively). At the same time an abnormally high extracellular space was detected (35–45 and 70–75% of total water, after 1 or 2 hr incubation, respectively); this was accounted for by an increase in the serosal extracellular space, confirmed by the addition of phenol red (or PEG 4000) to the serosal solution only. Since we considered that under the last two conditions the extracellular space was overestimated (see Discussion), we did not calculate the cell Na, K, and water. Under control conditions or with serosal ouabain the extracellular space was always about 20% of total water.

After preincubation serosal Na concentration was 142 ± 0.4 m*M* under control conditions, 141 ± 0.6 m*M* in the presence of 20 m*M* ouabain, and 140 ± 1.0 and 141 ± 0.8 m*M* under the two other experimental conditions. Sodium transport into the serosal compartment under four experimental conditions is represented in Fig. 1. Under control conditions sodium transport was high and, after 20 min incubation, constant for the remaining experimental period; a reduction of this transport to about one-fourth, constant in time, was evident when 20 m*M* ouabain was present in the serosal compartment of the intestine. When ethacrynate or DNP plus MIA was also present in the incubating fluid Na transport stopped after 20 min. Since we considered that under this condition the measured extracellular space was an overestimate due to the entrance of phenol red into the cell (see Discussion) and

TABLE I. WATER AND ELECTROLYTE CONCENTRATIONS IN THE JEJUNAL ENTEROCYTE INCUBATED *IN VITRO*

	Wet wt/dry wt	Cell water ($ml \cdot g^{-1}$)	Cell Na ($meq \cdot liter^{-1}$)	Cell K ($meq \cdot liter^{-1}$)
Control				
Time zero	5.9 ± 0.2	3.9 ± 0.1	40 ± 2	106 ± 1
1 hr exp.	6.1 ± 0.1	3.8 ± 0.2	38 ± 4	110 ± 5
2 hr exp.	6.2 ± 0.3	4.0 ± 0.2	42 ± 3	105 ± 4
20 m*M* ouabain (serosal side)				
Time zero	5.3 ± 0.3	3.5 ± 0.2	51 ± 3***	99 ± 2**
1 hr exp.	5.4 ± 0.4	3.4 ± 0.2	106 ± 5***	40 ± 5***
2 hr exp.	5.4 ± 0.2	3.4 ± 0.3	108 ± 3***	33 ± 4***

Note. All data were obtained with the jejunal tract of the intestine everted and incubated at 28°C. Mean values ± SE are reported. Number of experiments is five. *P* values are versus the corresponding ones for the control (***<0.001; **<0.05; if no *, NS).

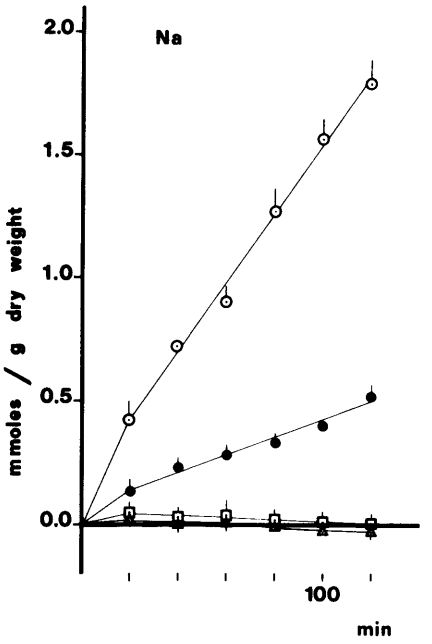

FIG. 1. Na transport into the serosal compartment (ordinate, mean values ± SE = bars) is reported during 2 hr incubation (abscissa, incubation time) under four experimental conditions: (1) Krebs–Ringer bicarbonate solution incubating fluid (open circles); (2) as (1) plus 20 m*M* ouabain in the serosal fluid (solid circles); (3) as (2) plus 2 m*M* ethacrynate in both incubating fluids (triangles); (4) as (2) plus 0.1 m*M* DNP and 3 m*M* MIA in both incubating fluids (squares).

since phenol red dilution was the parameter used to calculate all transports, these latter, in the presence of ethacrynate and DNP plus MIA, were corrected by subtracting the volume corresponding to the extracellular space exceeding 20% of total water.

The experimental conditions of the results reported in Fig. 2 are the same as those in Fig. 1 but here the transport of fluid into the serosal compartment is shown. Also in this case a high and constant transport was evident under control conditions; a reduction, constant in time, was observed in the presence of serosal ouabain; a further reduction and block of the transport after 20–40 min incubation occurred when ethacrynate or DNP plus MIA was added. As shown in Figs. 1 and 2, the results obtained with ethacrynate or DNP plus MIA were not significantly different.

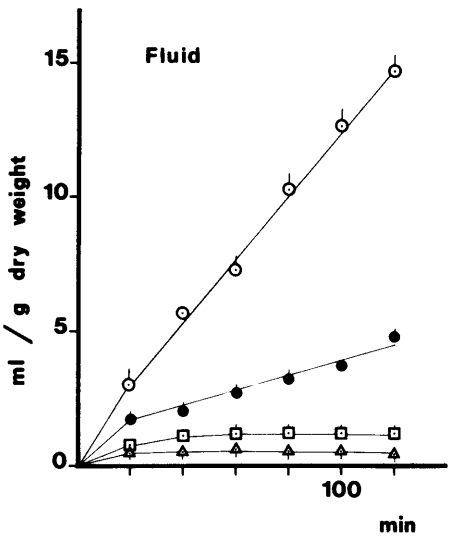

FIG. 2. Fluid transport into the serosal compartment during 2 hr incubation. For symbols, see Fig. 1.

After preincubation serosal K concentration was 5.74 ± 0.09 m*M* under control conditions, 6.76 ± 0.15 m*M* in the presence of 20 m*M* ouabain, and 7.00 ± 0.23 and 7.14 ± 0.22 m*M* under the two other experimental conditions. In Fig. 3, K diffusion into the

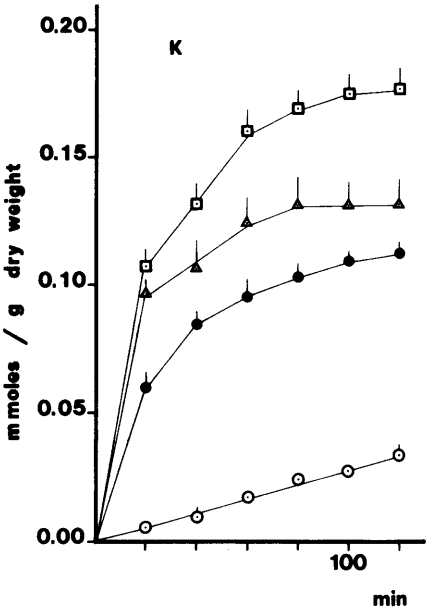

FIG. 3. K transport into the serosal compartment during 2 hr incubation. For symbols, see Fig. 1.

serosal compartment is reported: under control conditions a slight diffusion was observed in 2 hr incubation; this was dramatically increased by ouabain and still more by ethacrynate. A similar pattern was evident in the presence of DNP plus MIA only for 80 min, after which diffusion stopped.

Lactate transport into the serosal compartment is shown in Fig. 4. After 20 min incubation a high and constant transport of lactate was observed under control conditions. The same pattern, but with lower values, was obtained with ouabain in the serosal compartment, and also with ethacrynate. After only 20 min incubation lactate was no longer detected in the serosal compartment when DNP plus MIA was present in the incubation fluid.

Discussion. The results of *in vitro* jejunum experiments showed that wet weight/dry weight ratios, as well as cell water, Na and K concentrations, were constant during the 2-hr incubation under control conditions (Table I). The transport of Na and water into the serosal fluid after the first 20-min period was also constant for 2 hr under control conditions (Figs. 1 and 2). These findings, in addition to the simultaneous diffusion of K and lactate into the serosal compartment, the former low and the latter high but both constant in time (Figs. 3 and 4), seem to demonstrate that under control conditions, i.e., when the intestine is everted and incubated at 28°C with Krebs–Ringer bicarbonate solution, gassed with 95% O_2–5% CO_2 and with added 5.56 m*M* glucose, transport activities and electrolytes content of the enterocyte are in equilibrium. Although there is no blood supply, the tissue is oxygenated sufficiently because at 28°C the level of energy-rich nucleotides is constant (10, 11). It is known that the intestine incubated *in vitro* produces lactic acid even when maximally oxygenated, about one-half of which appears in the serosal solution (12).

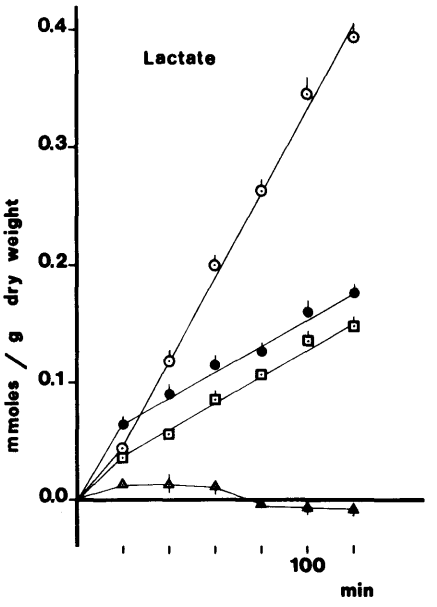

FIG. 4. Lactate transport into the serosal compartment during 2 hr incubation. For symbols, see Fig. 1.

To inhibit the Na–K pump, a high concentration of ouabain (20 m*M*) was used to ensure a complete block, also considering that higher concentrations are usually needed for rat intestine than for other tissues such as hamster intestine (13) or erythrocytes. When 20 m*M* ouabain was present in the serosal fluid, the cell gained Na and lost K, mostly during the first hour (Table I), and the wet weight/dry weight ratios and cell volumes were not significantly different from control values. During the subsequent 2 hr these parameters remained constant.

A dramatic rise in wet weight/dry weight ratios was obtained when ethacrynate or DNP plus MIA was present in the incubation fluid. Usually an increase in this parameter parallels an increase in cell volume (11); on the contrary we found that the extracellular space was abnormally high (and largely serosal). However, a decrease in transport activity normally causes a cell swelling (11). In our study the experimental conditions blocked all active processes. Therefore, it is evident that the increased extracellular space was not real but due to the entrance of phenol red into the cells, whose membranes lost their integrity. The possibility that phenol red can also enter the cell under control conditions, due to the insufficiently high molecular weight, can be excluded because during the 2-hr incubation the extracellular space did not vary. Furthermore, by using PEG of mol wt 4000 (9), the cell volume was slightly higher and the extracellular space slightly lower but, as in the case of phenol red, the two values were constant for 2 hr.

In addition to other inhibitory effects, ethacrynate has been described as a strong inhibitor of ouabain-insensitive Na–ATPase in the small intestine (4) and as blocking the ouabain-insensitive Na pump (7). It has been suggested that the main function of the ouabain-insensitive Na pump is the regulation of cell volume (6). Our results can be interpreted in this light. In fact, when the Na–K pump was blocked by ouabain, the cell volume and wet weight/dry weight ratio remained constant with respect to the control values, whereas when both pumps were without fuel (ATP production blocked by the uncoupling reagent DNP and by the inhibitor of anaerobic glycolysis MIA) and when the ouabain-insensitive Na pump was also blocked by ethacrynate, the wet weight/dry weight ratio increased remarkably.

Figure 4 shows that the lactate detected in the serosal compartment of the intestine was constant under control conditions. The inhibition of lactate production in the presence of ouabain is probably caused by poor activation of pyruvate kinase due to the low level of cell potassium; this phenomenon is enhanced by ethacrynate, perhaps as a result of increased release of K from the cell. The absence of lactate production for most of the 2-hr incubation in the presence of MIA shows that under this condition ATP production by anaerobic glycolysis is absent.

In general, when the Na–K pump is blocked by ouabain, diffusion of Na from the serosal fluid into the cell is expected due to the favorable electrochemical gradient. On the contrary, we found that in the presence of 20 m*M* ouabain the positive transport of both Na and fluid into the serosal compartment, although low, lasted for 2 hr. These results could suggest that the ouabain-insensitive Na pump is active at the serosal pole of the cell and that the net positive transport of Na into the serosal fluid is the difference between the activity of the ouabain-insensitive Na pump and the passive flux of Na from the serosal fluid into the cell. Certainly the activity of this pump is not sufficient to pump out all the Na, which also enters the cell from the brush border, so the cell gains Na. With the addition of ethacrynate or DNP plus MIA the transport of both Na and fluid was already blocked after a short time (Figs. 1 and 2). The very similar results obtained under the two experimental conditions indicate that in both cases an active process is inhibited. Therefore, these results suggest the existence of a ouabain-insensitive Na pump in intact tissue.

From the data in Fig. 3 it is evident that in the presence of ouabain K diffusion into the serosal solution is much higher than control values, as it is to be expected. Furthermore, after 2 hr a downhill K gradient was still present. The greater K flux in the presence of ethacrynate could be explained by a membrane damage, as previously suggested. The flux in the presence of DNP plus MIA stopped at 80 min; the reason for this is still unclear.

Preliminary results showed that the transintestinal electrical P.D., even if 1–1.5 mV lower with respect to the control condition, was always serosal positive during the 2-hr incubation in the presence of 20 m*M* ouabain.

K diffusion from the cell toward the serosal fluid could drive Na passively if Na–K (2 Cl) cotransport was present in the basolateral membrane. This possibility can be excluded for two reasons. First, as far as we know, this cotransport is not localized in the basolateral membrane. Second, the ratios between K and Na transported into the serosal compartment, obtained from data of Figs. 1 and 3, were very different under different experimental conditions.

In conclusion, the results of this study are easily explained by the existence of a ouabain-insensitive Na pump, which has been already demonstrated in guinea pig intestine (4, 7) and kidney (1, 2) and in rat kidney (3, 5, 6). The regulatory function of cell volume performed by this Na pump was evident when the Na–K pump was blocked by ouabain.

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1. Proverbio F, Del Castillo JR. Na^+-stimulated ATPase activities in kidney basal-lateral plasma membranes. Biochim Biophys Acta **646**:99–108, 1981.
2. Del Castillo JR, Marin R, Proverbio T, Proverbio F. Partial characterization of the ouabain-insensitive,

Na^+-stimulated ATPase activity of kidney basal-lateral plasma membranes. Biochim Biophys Acta **692**:61–68, 1982.

3. Proverbio F, Proverbio T, Marin R. Na^+–ATPase is a different entity from the (Na^+ + K^+)-ATPase in rat kidney basolateral plasma membranes. Biochim Biopys Acta **858**:202–205, 1986.
4. Del Castillo JR, Robinson JWL. Na^+-stimulated ATPase activities in basolateral plasma membranes from guinea-pig small intestinal epithelial cells. Biochim Biophys Acta **812**:413–422, 1985.
5. Marin R, Proverbio T, Proverbio F. Active sodium transport in basolateral plasma membrane vesicles from rat kidney proximal tubular cells. Biochim Biophys Acta **814**:363–373, 1985.
6. Marin R, Proverbio T, Proverbio F. Effect of Ca^{2+} on the ouabain-insensitive, active Na^+ uptake in inside-out basolateral plasma membrane vesicles from rat kidney proximal tubular cells. Biochim Biophys Acta **817**:299–306, 1985.
7. Del Castillo JR, Robinson JWL. Mg^{2+}–ATP-dependent sodium transport in inside-out basolateral plasma membrane vesicles from guinea-pig small intestinal epithelial cells. Biochim Biophys Acta **812**:402–412, 1985.
8. Esposito G, Faelli A, Tosco M, Orsenigo MN, Battistessa R. Age-related changes in rat transintestinal transport of D-glucose, sodium and water, Amer J Physiol **249**:G328–G334, 1985.
9. Esposito G, Faelli A, Tosco M, Burlini N, Capraro V. Extracellular space determination in rat small intestine by using markers of different molecular weights. Pfluegers Arch **382**:67–71, 1979.
10. Faelli A, Esposito G, Capraro V. Energy-rich phosphates and transintestinal transport in rat intestine incubated in vitro at different temperatures. Biochim Biophys Acta **455**:759–766, 1976.
11. Faelli A, Esposito G, Burlini N, Tosco M, Capraro V. The rat and hamster jejunum during transintestinal transport in vitro. Arch Int Physiol Biochem **87**:73–86, 1979.
12. Wilson TH, Wiseman G. The use of the sac of everted small intestine for study of the transference of substances from the mucosal to the serosal surface. J Physiol (London) **123**:116–125, 1954.
13. Robinson JWL. The difference in sensitivity to cardiac steroids of (Na^+ + K^+)-stimulated ATPase and amino acid transport in the intestinal mucosa of the rat and other species. J Physiol (London) **206**:41–60, 1970.

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