J. Membrane Biol. 120, 201–210 (1991)
002226319100047W

The Journal of
Membrane Biology
© Springer-Verlag New York Inc. 1991

Na,K-ATPase and the Development of Na^+ Transport in Rat Distal Colon

J. Pácha, J. Teisinger, M. Popp, and K. Čapek
Institute of Physiology, Czechoslovak Academy of Sciences, CS-142 20, Prague 4-Krč, Vídeňská 1083, Czechoslovakia

Summary. Na,K-ATPase function was studied in order to evaluate the mechanism of increased colonic Na^+ transport during early postnatal development. The maximum Na^+-pumping activity that was represented by the equivalent short-circuit current after addition of nystatin (I_{sc}^N) did not change during postnatal life or after adrenalectomy performed in 16-day-old rats. I_{sc}^N was entirely inhibited by ouabain; the inhibitory constant was 0.1 mM in 10-day-old (young) and 0.4 mM in 90-day-old (adult) rats. The affinity of the Na,K pump for Na^+ was higher in young (11 mM) than in adult animals (19 mM). The Na,K-ATPase activity (measured after unmasking of latent activity by treatment with sodium dodecylsulfate) increased during development and was also not influenced by adrenalectomy of 16-day-old rats. The inhibitory constant for ouabain (K_I) was not changed during development (0.1–0.3 mM). Specific [^{3}H]ouabain binding to isolated colonocytes increased during development (19 and 82 pmol/mg protein), the dissociation constant (K_D) was 8 and 21 μM in young and adult rats, respectively. The Na^+ turnover rate per single Na,K pump, which was calculated from I_{sc}^N and estimated density of binding sites per cm^2 of tissue was 500 in adult and 6400 Na^+/min · site in young rats. These data indicate that the very high Na^+ transport during early postnatal life reflects an elevated turnover rate and increased affinity for Na^+ of a single isoform of the Na,K pump. The development of Na^+ extrusion across the basolateral membrane is not directly regulated by corticosteroids.

Key Words Na,K-ATPase · Na^+ transport · ouabain binding · nystatin · corticosteroids · development · rat distal colon

Introduction

The transepithelial Na^+ transport in the rat distal colon involves passive Na^+ movement across the apical membrane and subsequent active Na^+ extrusion across the basolateral membrane by the Na,K pump [29]. There is abundant experimental evidence that adrenal corticosteroids increase and adrenalectomy decreases Na^+ transport and the permeability of the apical membrane of colonocytes to Na^+ [2, 3, 6, 29, 41]. The corticosteroids increase Na^+ flux into the cell which in turn stimulates the active Na^+ extrusion via the Na,K pump, because the intracellular Na^+ activity remains approximately constant in spite of the manifold variations of Na^+ current across the epithelium [38]. There are, in addition, indications that the Na,K pump itself may be influenced directly by corticosteroids. Many authors reported that corticosteroids increase [3, 6, 20, 41] and adrenalectomy decreases [34] the activity of colonic Na,K-ATPase — the enzymatic equivalent of the Na,K pump.

In contrast to the fact that Na^+ transport and Na,K-ATPase activity are roughly proportional in the adult colon, developmental studies have demonstrated a temporal dissociation between the developmental patterns of Na^+ transport and Na,K-ATPase. Na,K-ATPase shows an age-dependent increase [11] similar to other epithelia [8], although Na^+ absorption is very high during the suckling period and significantly decreases around the time of weaning [11]. The very high Na^+ absorption is a consequence of electrogenic amiloride-sensitive Na^+ transport which is very high during the suckling period, disappears after weaning and is induced by adrenal corticosteroids [26, 27]. The presence of this pathway during neonatal life contrasts with the findings in the distal colon of adult rats where Na^+ is transported predominantly via an electroneutral Cl^--dependent mechanism [29] and where the electrogenic amiloride-sensitive pathway can be induced only by secondary hyperaldosteronism or by treatment with pharmacological doses of corticosteroids [16, 40].

Considering that Na^+ transport is crucially dependent on the Na,K pump [29], it is evident that the developmental patterns of Na^+ transport and Na,K-ATPase activity are controversial. Because ATP hydrolysis is usually measured under V_{max} conditions and Na^+ transport is not, we wondered whether the controversy about developmental patterns might be due to the different conditions during measurement or due to changes of properties of the transport system. To explore these questions we studied (*i*) the maximum Na^+-pumping activity

(maximum transport capacity) in the epithelium using the nystatin method, (*ii*) the hydrolytical activity of the enzyme, (*iii*) the number of Na,K-ATPase sites determined by the specific binding of [^{3}H]ouabain, and (*iv*) the sensitivity of the maximum Na^+-pumping activity, hydrolytical activity and [^{3}H]ouabain binding to ouabain.

Material and Methods

Treatment of Animals

Experiments were performed on suckling (10-day-old), weanling (20- and 30-day-old), and adult (90-day-old) rats that were kept on a standard diet (0.17 mmol Na^+/g and 0.20 mmol K^+/g) and tap water ad libitum. Approximately 24 hr postpartum all litters were reduced to eight or nine pups and housed with their mothers until the age of 30 days. In some experiments young rats were adrenalectomized four days before the experiments. After the surgery, the pups were returned to their mothers and drinking water was replaced by saline. In another series of experiments some adult rats with intact adrenal glands received i.p. injections of DOCA[1] (0.5 mg/100 g body wt per 12 hr) or dexamethasone (0.3 mg/100 g body wt per 12 hr) for four days before the experiments.

Measurement of Maximum Na^+-Pumping Activity

The distal colon was rinsed of the luminal content, stripped of the serosa and a part of muscle layers and mounted in an Ussing chamber. The epithelium was bathed on both sides with Na_2SO_4-Ringer solution at 37°C and continuously oxygenated (95% O_2 + 5% CO_2). Na_2SO_4-Ringer was used instead of NaCl-Ringer because Cl^- rapidly deteriorates the nystatin-treated preparation [42]. The composition of the solution was (in mM): 54.9 Na_2SO_4; 25.0 $NaHCO_3$, 2.4 Na_2HPO_4, 0.4 NaH_2PO_4, 1.2 $MgSO_4$, 1.2 Ca gluconate, 10.0 glucose, and 55.0 mannitol. I_{sc} was measured as described previously using an automatic voltage clamp which corrected for potential asymmetry of electrodes and fluid resistance [26, 27]. To determine the maximum Na^+-pumping activity the polyene antibiotic nystatin (dissolved in DMSO) was added to the mucosal solution in a concentration of 750 U/ml. This channel-forming drug markedly increased the apical membrane conductance and thus eliminated the rate-limiting factor for transepithelial Na^+ transport under physiological conditions. The Na^+ delivery to the Na,K pump was increased to the level that saturated the pump mechanism and thus I_{sc} measured after this treatment is assumed to represent the transport mediated by the Na,K pump [12, 16, 42]. Preliminary experiments had shown that the amount of DMSO added to the bathing solution (never greater than 0.3%) had no significant effect on I_{sc} within the measured time period. In the experiments where ouabain sensitivity of I_{sc}^N was examined, ouabain was added to the serosal solution 30–45 min before the application of nystatin. For the measurement of Na,K pump activation by Na^+, the colon was incubated in K_2SO_4-Ringer solution in which K^+ was substituted for Na^+. After the addition of nystatin, subsequent aliquots of Na_2SO_4 were added first to the serosal solution where they had no effects and then to the mucosal solution. In another set of experiments I_{sc}^A was measured after addition of amiloride (10^{-4} M) to the mucosal solution.

Measurement of Na,K-ATPase Activity

Na,K-ATPase activity was measured in crude homogenates by determination of ouabain-inhibitable release of inorganic phosphate from ATP. The mucosa obtained from the distal colon by scraping with a glass slide was homogenized with a Teflon pestle in an ice-cold solution containing (in mM): 30 Tris-HCl, 250 sucrose, 5 Na_2EDTA, pH 7.3. The homogenate (1.2–1.7 mg protein/ml) was then preincubated for 30 min with a detergent (DOC, SDS, Triton X-100, or Nonidet P-40) at room temperature. Samples of detergent preincubated homogenate were incubated at 37°C for 10 min in a solution containing (in mM): 100 NaCl, 100 Tris-HCl, 20 KCl, 5 $MgCl_2$, pH 7.3, without or with various concentrations of ouabain. The reaction was started by the addition of ATP (final concentration of 3.2 mM) and continued for 30 min. The concentration of protein in the assay was 60–90 μg/ml and the total volume was 1 ml. After stopping the reaction by addition of 0.25 ml ice-cold TCA, the released inorganic phosphate was assayed according to Taussky and Shorr [37]. Na,K-ATPase activity was calculated as the difference between ATPase activity without and with ouabain (2×10^{-3} M) and expressed as μmol of inorganic phosphate per mg of protein per hour. Protein concentrations were determined by the method of Lowry et al. [23] using bovine serum albumin (Fraction V, Sigma) as a standard. All measurements were performed in triplicate.

Measurement of Number of Na,K Pumps

The concentration of ouabain binding sites was measured in isolated colonocytes using a [^{3}H]ouabain displacement assay [1]. The cells were isolated by a modification of the methods of Morris, Gallacher and Lee [25] and Rowling and Sepúlveda [32]. The distal colon of adult rats was removed and washed with saline containing 1 mM dithiothreitol to remove adherent mucus, then the colon was turned inside out, tied on one end, filled with saline (under a slight pressure) and tied on the other side. The colon preparations made in this manner were incubated at 37°C in a solution consisting of (in mM): 96.0 NaCl, 27.0 Na citrate, 1.5 KCl, 5.6 Na_2HPO_4, 1.8 KH_2PO_4; pH 7.4. After 10-min incubation the colons were placed for 10 min in HEPES buffer (in mM: 120.0 NaCl, 5.0 KCl, 1.5 $MgCl_2$, 10.0 glucose, 10.0 Na pyruvate, 1.0 dithiothreitol, 10.0 ascorbic acid, 20.0 HEPES, 1.5 Na_2EDTA, and albumin 1 mg/ml, pH 7.4) and finally for 10 min in HEPES buffer which contained hyaluronidase 1.5 mg/ml. After incubation the colons were transferred to hyaluronidase-free, ice-cold HEPES buffer where colonocytes were released by manual stirring. The cells were filtered through nylon mesh (100 and 75 μm), washed twice and resuspended in a K^+-free buffer of the following composition (in mM): 143.0 NaCl, 1.5 MgCl, 4.2 $NaHCO_3$, 5.5 glucose, 5.0 Na pyruvate, 10.0 HEPES, pH 7.2. The mixture

[1] *Abbreviations used:* B_{max}, maximum binding of [^{3}H]ouabain; DMSO, dimethyl sulfoxide; DOC, sodium deoxycholate; DOCA, deoxycorticosterone acetate; HEPES, N-2-hydroxyethyl piperazine-N′-2-ethanesulfonic acid; I_{sc}, short-circuit current; I_{sc}^A, amiloride-sensitive short-circuit current; I_{sc}^N, short-circuit current in the presence of nystatin; K_D, dissociation constant of [^{3}H]ouabain binding; K_I, inhibitory constant; Na_2EDTA, ethylenediaminetetraacetic acid, disodium salt; SDS, sodium dodecylsulfate; TCA, trichloracetic acid; Tris, tris(hydroxymethyl)aminomethane.

contained 1.7–2.3 mg TCA-precipitable protein/ml. The isolation of colonocytes from young animals followed nearly the same procedure but distal colons were mounted as flat sheets on a plastic holder (mucosa upwards). Cell viability was tested by Trypan blue at the end of the isolation procedure, and no differences between cells from young and adult animals were found.

As Na,K-ATPase can bind ouabain only in its phosphorylated configuration, we used the K^+-free buffer and vanadate for the binding assay. The latter is bound to the phosphorylation site with higher affinity than ATP [18]. Aliquots of cell suspension were resuspended at 37°C in 400 μl of incubation solution containing K^+-free buffer, 1 mM Na_3VO_4, and albumin 1 mg/ml without or with various concentrations of nonradioactive ouabain. After the preincubation of cells for 10 min the binding assay was initiated by adding [^{3}H]ouabain (0.8 μCi/ml incubation medium). Incubation was continued for 90 min and then 3 × 5 ml of ice-cold 10 mM Tris-HCl were added to the cells, and the mixture was filtered through a glass microfiber filter, Whatman GF/C. The filter was extracted in liquid scintillator SLD-41, and radioactivity was counted using a liquid scintillation counter. As nonspecific ouabain binding was expected, the binding was also studied in the above-mentioned medium including 5×10^{-3} M of nonlabeled ouabain. Results were expressed in fmol of bound [^{3}H]ouabain per mg cell protein. Cell protein was determined by the precipitation of aliquots of cell suspension by TCA followed by centrifugation at 10.000 × g for 15 min. The pellets were solubilized in 1 N NaOH and protein analyzed by the method of Lowry et al. [23]. Na,K-ATPase assay of isolated cells was similar to measurement of Na,K-ATPase activity in epithelial homogenate. Twice washed colonocytes were resuspended in Tris-buffer and homogenized. The subsequent procedure was the same as mentioned above.

Chemicals and Statistics

Amiloride was obtained from Merck, Sharp & Dohme (Hoddesdon, UK); DOC, DOCA, Triton X-100, SDS, nystatin, ouabain, HEPES, Tris, bovine serum albumin, ATP, hyaluronidase, DMSO, and calcium gluconate from Sigma (St. Louis, MO); dexamethasone from Spofa (Prague, Czechoslovakia); [^{3}H]ouabain from Amersham (UK); and liquid scintillator SLD-41 from Spolana (Neratovice, Czechoslovakia). All others chemicals were purchased from Labora (Brno, Czechoslovakia).

Unless otherwise stated the values were expressed as means ± SEM. Differences between groups were determined by the Student's unpaired t test. A value of $P < 0.05$ was accepted to indicate statistical significance. Curve fittings were performed using nonlinear regression analysis of means which were calculated from values obtained in individual experiments. The parameters of the models were estimated by nonlinear least squares. The following method was used to compare parameters between two independent experiments. Parameters p_1 and p_2 were different at the 5% confidence level if confidence intervals $\hat{p}_1 \pm 2\,\widehat{\text{SD}}_1$ and $\hat{p}_2 \pm 2\,\widehat{\text{SD}}_2$ did not intersect; $\hat{p}_1$ and $\hat{p}_2$ are estimates of parameters p_1 and p_2 and $\widehat{\text{SD}}_1$ and $\widehat{\text{SD}}_2$ their estimated standard errors [9].

Results

Maximum Pumping Activity of the Sodium Pump

To compare hydrolytic activity of Na,K-ATPase measured in broken cell preparations under V_{max} conditions with its maximum activity in intact cells

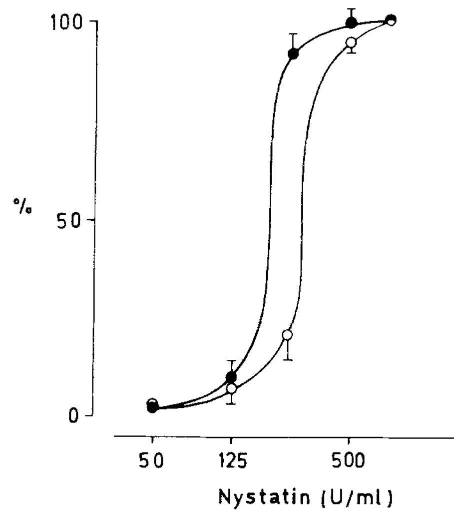

Fig. 1. Dose-response curves demonstrating the effect of mucosal nystatin on the short-circuit (I_{sc}) in the distal colon of adult (—○—) and 20-day-old rats (—●—). The preparations were incubated in Na_2SO_4-Ringer solution, and the concentration of nystatin was increased by subsequent addition of nystatin dissolved in dimethyl sulfoxide. %: mean percentage of maximum nystatin-stimulated I_{sc}; data points are means ± SEM of six experiments. The curves were drawn by eye

I_{sc}^N was investigated. Figure 1 shows that I_{sc} increased after the addition of nystatin in a dose-dependent manner and that the maximally effective concentration was 750 U/ml. This concentration was, therefore, used in all other experiments. To verify that I_{sc}^N can be used as an estimate of Na^+-pumping activity of the Na,K pump we studied I_{sc}^N under conditions when Na^+ transport as well as Na,K-ATPase activity were increased or decreased. DOCA in a dose that stimulated Na^+ transport and Na,K-ATPase activity in the rat colon [6] significantly increased I_{sc}^N from 345 ± 40 μA/cm^2 (n = 17) in adult controls to 730 ± 93 μA/cm^2 (n = 7) in DOCA-treated rats ($P < 0.05$). The same twofold increase was found after dexamethasone (717 ± 78 μA/cm^2; n = 5; $P < 0.01$) which is also known to stimulate Na^+ transport and Na,K-ATPase activity [3]. Similar values of I_{sc}^N were recently demonstrated by Halevy et al. [16] in the distal colon of rats with secondary hyperaldosteronism. For the depression of Na^+-pumping activity we used ouabain, an inhibitor of Na,K-ATPase activity [18] and Na^+ transport [5]. Dose-response curves of the ouabain effect on I_{sc}^N are shown in Fig. 2. The curves were calculated using a model equation:

$$I_{sc}^N = (I_{sc}^N)_o + (I_{sc}^N)_{max}/(1 + [I]/K_I) \qquad (1)$$

where I_{sc}^N and $(I_{sc}^N)_{max}$ were short-circuit currents in the presence and absence of ouabain, $[I]$ was the

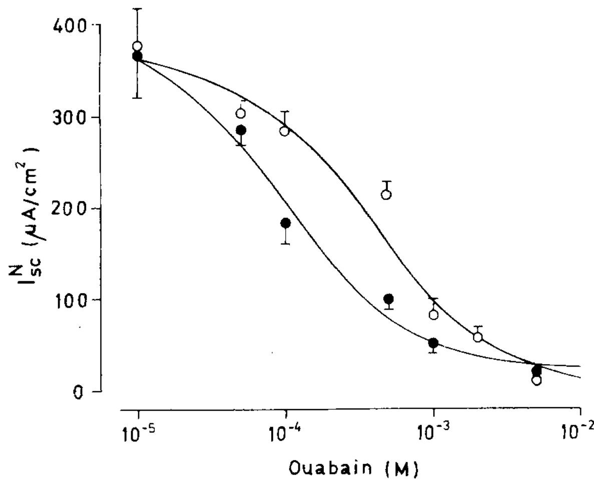

Fig. 2. Ouabain inhibition of the short-circuit current after mucosal application of nystatin (I^N_{sc}) in 90-day-old (—○—) and 10-day-old rats (—●—). Preparations of the distal colon were incubated in Na_2SO_4-Ringer solution containing various concentrations of ouabain on the serosal side. After stabilization of the I_{sc}, nystatin was added to the mucosal side to reach the final concentration of 750 U/ml. Each data point represents the mean ± SEM of 5–9 experiments. The curves were plotted for a single-site binding model by nonlinear regression analysis according to Eq. (1). Adult rats: $(I^N_{sc})_o$ = 0 and $(I^N_{sc})_{max}$ = 367 ± 24 μA/cm^2; K_I = 4.2 ± 1.1 × 10^{-4}M; young rats: $(I^N_{sc})_o$ = 20 ± 16 and $(I^N_{sc})_{max}$ = 375 ± 23 μA/cm^2; K_I = 1.0 ± 0.2 × 10^{-4} M; values are parameters ± asymptotic SD

Table 1. Effects of nystatin and amiloride on the short-circuit current in the rat distal colon during development

	Age (days)			
	10	20	30	90
I_{sc}	274 ± 41	368 ± 48	92 ± 13	51 ± 13
I^N_{sc}	393 ± 51	438 ± 38	323 ± 60	345 ± 40
n	15	10	8	17
I^A_{sc}	205 ± 37	379 ± 73	75 ± 12	0
n	7	6	5	7

Values are means ± SEM. I_{sc} is the short-circuit current before and I^N_{sc} after the addition of nystatin, I^A_{sc} is the amiloride-sensitive part of the short-circuit current, values are given in μA/cm^2; n = number of animals.

concentration of ouabain and K_I the inhibitory constant, $(I^N_{sc})_o$ was a constant parameter which represents ouabain-insensitive I_{sc}. It can be seen that ouabain significantly decreased I^N_{sc} nearly to zero and K_I was very similar in both groups.

The development of maximum Na^+-pumping activity was examined in the distal colon of suckling, weanling, and adult rats. This is shown in Table 1

Table 2. Influence of adrenalectomy on the effect of nystatin and amiloride on the short-circuit current in the distal colon of 20-day-old rats

	I_{sc}	I^N_{sc}	I^A_{sc}
Controls	368 ± 43 (10)	439 ± 38 (10)	370 ± 73 (6)
Adrenalectomy	165 ± 25 (8)[a]	486 ± 44 (8)	14 ± 7 (8)[a]

Values are means ± SEM; numbers of animals are given in parentheses. Values are given in μA/cm^2. For the symbols, *see* Table 1. Adrenalectomy was performed four days before the experiments.
[a] Significantly different from the controls ($P < 0.01$).

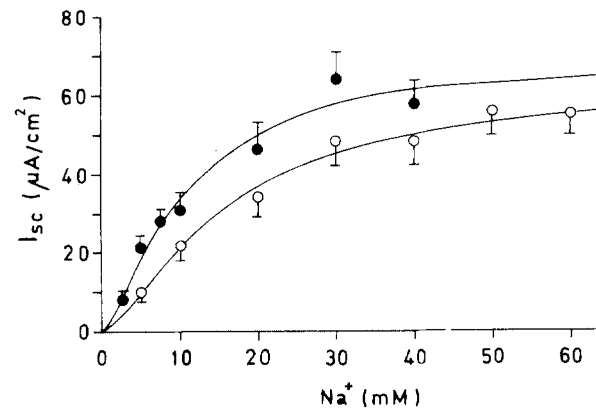

Fig. 3. A plot of mucosal Na^+ concentration *versus* short-circuit current (I_{sc}) in the distal colon of 90-day-old (—○—; n = 9) and 10-day-old rats (—●—; n = 10). Preparations of the distal colon were incubated in K_2SO_4-Ringer solution containing nystatin to increase the apical permeability for monovalent cations. The Na^+ concentration was increased by subsequent addition of small aliquots of Na_2SO_4 to both sides of the epithelium. Each data point represents the mean ± SEM of 10 experiments. The curves were plotted according to Eq. (2) by nonlinear regression analysis. Adult rats: I^{max}_{sc} = 71 ± 3 μA/cm^2; K_{Na} = 4.9 ± 0.5 mM; young rats: I^{max}_{sc} = 75 ± 7 μA/cm^2; K_{Na} = 2.9 ± 0.5 mM; values are parameters ± asymptotic SD

together with the values of I^A_{sc} which represent the electrogenic amiloride-sensitive Na^+ transport through Na^+ channels. Comparison of I^N_{sc} and I^A_{sc} shows that I^A_{sc} is and I^N_{sc} is not age dependent. In our earlier study [26, 27] I^A_{sc} was found to be induced by corticosteroids. To ascertain whether Na^+-pumping activity was also corticosteroid dependent, we compared I^N_{sc} in control and adrenalectomized young rats (Table 2). The values of I^N_{sc} were not changed after adrenalectomy even if I^A_{sc} disappeared. Taken together these results indicate that maximum Na^+-pumping activity is independent of age and of the direct effect of corticosteroids.

To ascertain whether there are changes in the pump characteristics a kinetic study was performed to estimate the affinity of the Na,K pump for Na^+ (Fig. 3). The curves were calculated using an equa-

tion derived from Michaelis-Menten kinetics with three noninteracting Na^+ binding sites which gave better fit than the model of highly cooperative sites [17]. The noninteracting model equation was

$$I_{sc} = I_{sc}^{max}/(1 + K_{Na}/[Na^+])^3 \quad (2)$$

where I_{sc} was the value of the short-circuit current at various concentrations of Na^+, I_{sc}^{max} the maximal value of I_{sc}, K_{Na} the substrate concentration at which 50% of binding sites were occupied and $[Na^+]$ the mucosal Na^+ concentration. The $K_{0.5}$, i.e., the substrate concentration at which the Na^+-pumping activity was half-maximal, could easily be calculated from the value of K_{Na} as: $K_{0.5} = K_{Na}/(\sqrt[3]{2} - 1) = 3.85\ K_{Na}$. Though there was no apparent change in I_{sc}^{max}, the affinity for Na^+ decreased with age ($P < 0.05$); $K_{0.5}$ being 11 mM for young and 19 mM for adult rats. These findings indicate that, at physiological intracellular sodium concentrations, Na^+ transport may operate at a higher rate in young rats than in adult animals. A comparison of the values of Na^+-pumping activity in K_2SO_4-Ringer (Fig. 3) and in Na_2SO_4-Ringer (Table 1) indicates that K_2SO_4-Ringer decreased the maximum activity. As nystatin pores are permeable for K^+ ions we assume that intracellular K^+ was increased in the presence of 145 mM extracellular K^+ and that intracellular K^+ inhibited Na^+-pumping activity as in red blood cells [13]. It is also possible that in the presence of K_2SO_4-Ringer Ca^{2+} extrusion via Na^+/Ca^{2+} countertransport was inhibited and that consequently the increased intracellular Ca^{2+} partially inhibited the Na,K-ATPase [44].

Development of Na,K-ATPase Activity

To avoid underestimation of Na,K-ATPase due to its latent activity [21, 32] the maximal activity was unmasked by means of various ionic and nonionic detergents (Fig. 4). All detergents had a biphasic effect. The activation by Triton X-100 and Nonidet P-40 was relatively small (30–50%), whereas the effects of DOC and SDS were much more pronounced (150–250%). As SDS caused the greatest unmasking of latent activity it was used in further experiments. Due to the age-dependent differences in the maximal effect, we used the concentration of 0.075 mg SDS/ml for adult and 0.2 mg SDS/ml for young rats (Fig. 4).

The Na,K-ATPase activity increased significantly ($P < 0.05$) during weaning and thereafter remained stable (Table 3). Adrenalectomy did not significantly influence the Na,K-ATPase activity in 20-day-old rats. Na,K-ATPase activity was 5.14 ± 0.17 μmol P_i/mg · hr ($n = 4$) in sham-operated controls and 4.95 ± 0.42 μmol P_i/mg · hr ($n = 6$) in adrenalectomized rats. Thus the Na,K-ATPase activity and

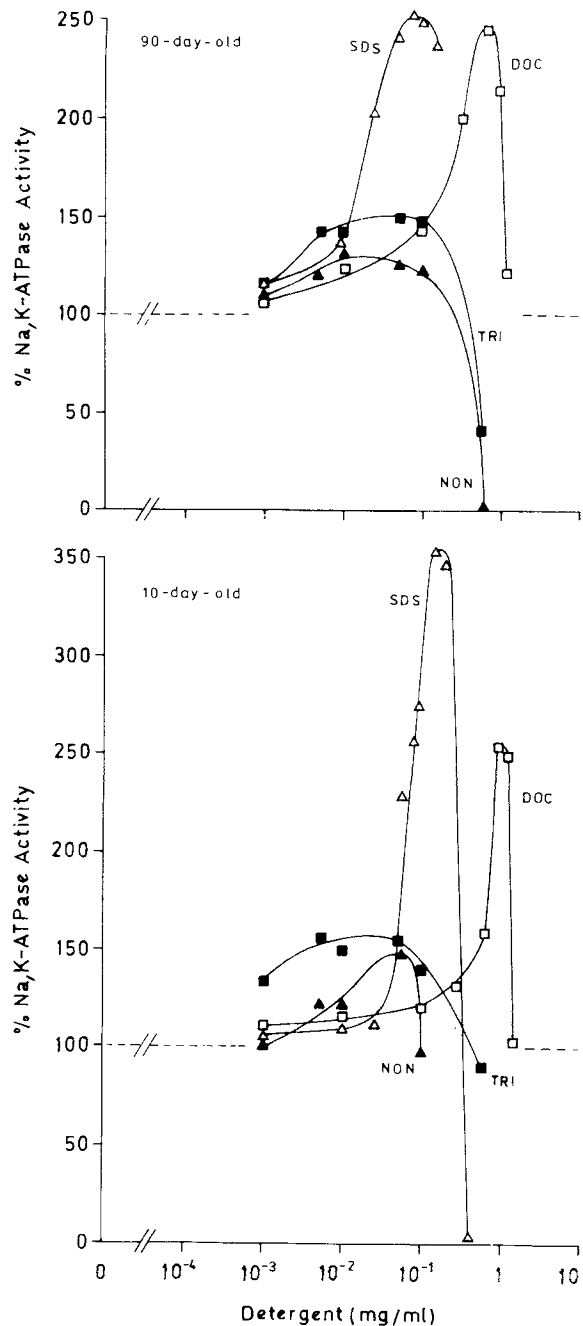

Fig. 4. Effect of deoxycholate (DOC), dodecylsulfate (SDS), Triton X-100 (TRI) and Nonidet P-40 (NON) on Na,K-ATPase activity of the distal colon of young (10-day-old) and adult (90-day-old) rats. The crude homogenate (1.2–1.7 mg protein/ml) was preincubated with various detergents at indicated concentrations for 30 min at room temperature and then assayed for Na,K-ATPase activity. All activities refer to the controls that were preincubated without any detergent. Each data point represents four experiments. The curves were drawn by eye

Table 3. Development of Na,K-ATPase activity in colonic mucosa

Age (days)	Na,K-ATPase activity (μmol P_i/mg · hr)	K_I (10^{-4} M)
10	3.32 ± 0.23 (16)	2.2 ± 0.6 (6)
20	3.95 ± 0.29 (8)	1.4 ± 0.2 (4)
30	5.19 ± 0.36 (8)[a]	2.7 ± 0.6 (4)
90	4.79 ± 0.32 (18)[a]	1.0 ± 0.2 (6)

Values are the means ± SEM (activity) or parameters ± asymptotic SD (K_I), numbers of experiments are given in parentheses. Homogenates were preincubated with dodecylsulfate 30 min at room temperature at a concentration of 1.2–1.7 mg protein/ml and then assayed for ATPase activity. Inhibitory constants (K_I) for ouabain were determined in another set of experiments. For further details, *see* Fig. 5.
[a] Significantly different from the 10-day-old rats ($P < 0.05$).

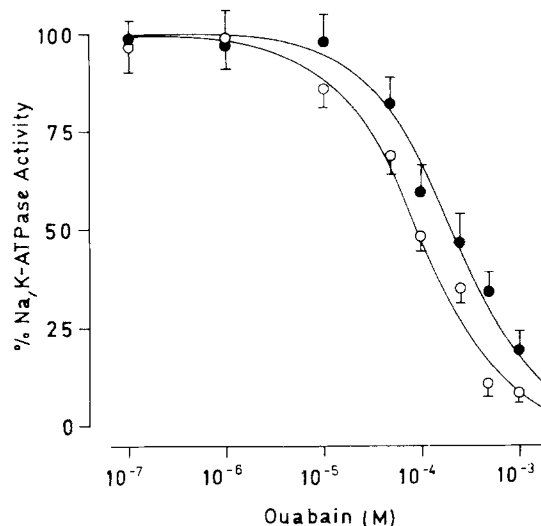

Fig. 5. Ouabain inhibition of Na,K-ATPase activity from the distal colon of 10-day-old (—●—) and 90-day-old rats (—○—). Homogenates were preincubated with dodecylsulfate (young; 0.2 and adult: 0.075 mg SDS/ml) and incubated with various concentrations of ouabain. Each data point represents the mean ± SEM of six experiments. The curves were plotted for a single-site binding model by nonlinear regression analysis according to Eq. (3). The values of K_I are given in Table 3

Table 4. Development of protein content in colonic mucosa

Age (days)	10	20	30	90
mg protein/cm²	6.0 ± 0.4	12.4 ± 0.9	12.5 ± 0.06	15.8 ± 1.0

Values are the means ± SEM. Colonic mucosa was scraped, homogenized, and assayed for protein.

the Na^+-pumping activity had opposite developmental patterns. To better understand these results we measured the protein content in colonic mucosa. The resulting data (Table 4) clearly illustrated that the protein content per cm² increased. Thus the difference in the development of I_{sc}^N and Na,K-ATPase activity was present even if we related the activity to the surface area.

To characterize the colonic Na,K-ATPase activity during development we compared its sensitivity to ouabain. As is shown in Fig. 5, ouabain induced a marked dose-dependent inhibition of Na,K-ATPase activity. The ouabain concentration causing 50% inhibition of Na,K-ATPase activity (K_I) was calculated according to the following equation:

$$V = V_{max}/(1 + [I]/K_I) \tag{3}$$

where V and V_{max} were the velocities of the hydrolytic reaction in the presence and absence of ouabain, respectively; $[I]$ the concentration of ouabain; and K_I the inhibitory constant. While activity increased during development K_I did not indicate any systematic changes (Table 3).

Pump Abundance and Na,K-ATPase Activity in Isolated Colonocytes

To resolve whether the developmental increase in the Na,K-ATPase activity is followed by an increase of the number of Na,K pumps, we studied the binding of [³H]ouabain to isolated colonocytes. Our preliminary experiments demonstrated that the steady-state level of binding increased with increasing concentration of [³H]ouabain. At the concentration of 4×10^{-8} M the steady-state was attained after 90 min. The specific [³H]ouabain binding calculated as the difference between total and nonspecific binding was a saturable process (Fig. 6). Nonspecific binding accounted for 27 and 25% of binding from young and adult rats, respectively. The parameters of binding were calculated according to Eq. (1):

$$B = B_{max}[^3HI]/(K_D + [^3HI] + [I]) \tag{4}$$

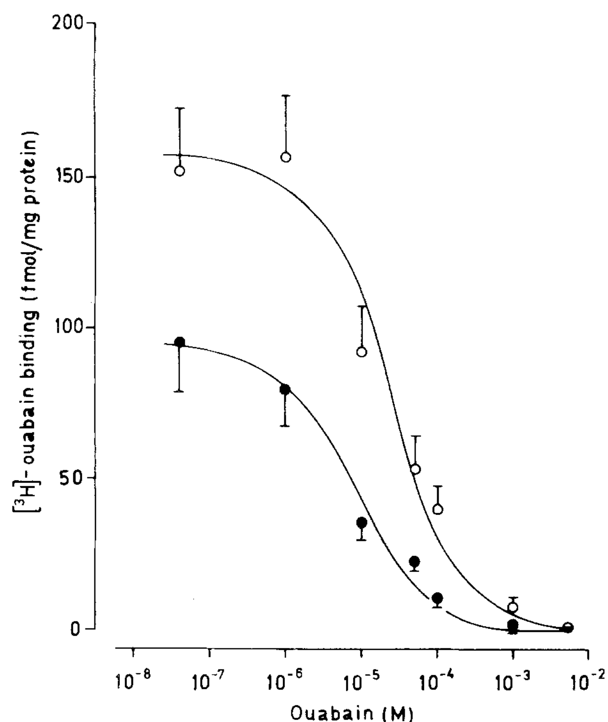

Fig. 6. Specific binding of [^{3}H]ouabain to isolated colonocytes of 10-day-old (-●-) and 90-day-old rats (-○-) in the presence of various concentrations of nonlabeled ouabain. Colonocytes were incubated with 40 nM [^{3}H]ouabain at 37°C for 90 min. All values were corrected for the nonspecific binding found in the presence of 5×10^{-3} M nonlabeled ouabain. Each data point represents the mean ± SEM of seven experiments. The curves were plotted according to Eq. (4) by nonlinear regression analysis. Adult rats: $B_{max} = 82 \pm 24$ pmol/mg protein; $K_D = 2.1 \pm 0.7 \times 10^{-5}$ M; young rats: $B_{max} = 19 \pm 6$ pmol/mg protein; $K_D = 0.8 \pm 0.2 \times 10^{-5}$ M; values are parameters ± asymptotic SD

where B was the binding of [^{3}H]ouabain at a given concentration of nonlabeled ouabain; B_{max} the maximum binding of [^{3}H]ouabain; [^3HI] and [I] the concentration of labeled and nonlabeled ouabain, respectively, and K_D the dissociation constant of binding, i.e., the concentration of ligand which produced a half-maximal ligand binding. The maximum binding capacity was significantly higher ($P < 0.05$) in adult animals, i.e., in the epithelium with a lower Na^+ transport. Using the data in Table 4, the total specific binding per cm^2 was 114 pmol/cm^2 in young and 1302 pmol/cm^2 in adult rats, respectively. This means that there are 69×10^{12} pump sites/cm^2 in young and 784×10^{12} in adult rats.

To compare the measured pump abundance in intact cells directly with their level of Na,K-ATPase activity, this activity was estimated enzymatically in cell samples. As in crude epithelial homogenate the Na,K-ATPase activity was significantly higher ($P < 0.05$) in 90-day-old rats (6.85 ± 0.48 μmol P_i/mg · hr; $n = 5$) than in 10-day-old animals (4.12 ± 0.31 μmol P_i/mg · hr; $n = 5$). The higher activity found in colonocytes in comparison with epithelial homogenates is the consequence of the preparation. After 30 min of isolation only the superficial, i.e., Na^+-transporting cells are harvested and no crypt cells which have two- to threefold lower activity of Na,K-ATPase [32]. The dissimilarity of the fractional increases in enzyme activity and abundance resulted in difference in the calculated maximal catalytic turnover number (transfer of inorganic phosphate per minute per enzyme molecule) which was 1400 in adult and 3600 in young rats.

Discussion

Our results indicate that the Na,K pump of the rat distal colon is not saturated with intracellular Na^+ when the epithelium is exposed to physiological concentrations of extracellular Na^+. Due to this fact, it is difficult to compare transport data with Na,K-ATPase activity which is measured under V_{max} conditions. The Na^+ permeability of the apical membrane is rate limiting for transepithelial Na^+ transport under physiological conditions. However, when this permeability is markedly increased by polyene antibiotics such as nystatin or amphotericin B, the rate of Na^+ transport becomes limited by the Na,K pump [12, 16, 17, 42]. We therefore used nystatin to measure Na^+ transport under V_{max} conditions. The assumption that I_{sc}^N can be used as a good estimate of maximum Na^+-pumping activity was supported by the sensitivity of I_{sc}^N to ouabain and corticosteroids.

As was demonstrated by Jørgensen and Skou [21] and Rowling and Sepúlveda [32], Na,K-ATPase activity in homogenates or in plasma membrane-rich fractions is partially masked probably because of the formation of closed vesicles during homogenization which hinders free access of substrates and activators to the respective membrane sites. In order to reveal this latent activity and to measure the maximal Na,K-ATPase activity we studied the effect of various detergents. Their effect was not the same and the concentrations resulting in maximal activation as well as the values of such activation also differed between young and adult animals. These differences may reflect the developmental changes in lipid composition and membrane fluidity [33]. Our results indicate that the unmasking effect is different in young and adult rats and that it should be considered in developmental studies.

Comparison of Na^+-pumping activity and Na,K-ATPase activity under V_{max} conditions, indicates different developmental patterns. During post-

natal development, the maximum Na^+-pumping activity was approximately constant, whereas Na,K-ATPase activity increased. There are at least four possible explanations for this: (*i*) Na,K pump has a rate-limiting step other than Na^+ permeability of the apical membrane in the presence of nystatin, (*ii*) in addition to the ouabain-sensitive Na,K pump there is also a ouabain-insensitive Na pump, (*iii*) there are developmental changes of Na,K-ATPase isoforms during maturation, or that (*iv*) there are changes in the kinetics and/or the number of pumps.

First, the addition of nystatin promotes the utilization of energy and the ability of mitochondria to produce ATP which may become the rate-limiting step in Na^+ transport under such conditions. Grasl, Krivanek and Turnheim [14], however, demonstrated that maximum Na^+-pumping activity measured in the presence of amphotericin B in the rabbit distal colon was not limited by ATP. Second, there is some evidence for the existence of a ouabain-insensitive Na^+ pump in the intestine [7]. However, if we compare the sensitivity of I_{sc}^N to ouabain (Fig. 2), we can eliminate the significance of such a pump in our experiments.

Third, developmental changes in isoforms of Na,K-ATPase with a different sensitivity for ouabain have been observed in some tissues [36] and this sensitivity probably correlated with differences in substrate affinities for Na^+ [24]. Our results indicate that colonocytes express only one isoform of functionally active Na,K-ATPase during all the developmental stages investigated. The inhibition of Na,K-ATPase activity and [^{3}H]ouabain binding by ouabain indicates the presence of a single class of low affinity sites as evidenced by the monophasic inhibition curves in all groups and by the absence of important changes in K_I and K_D during development. These data suggest that the colonic Na,K-ATPase is probably the ouabain-low-sensitive α-form similar to renal Na,K-ATPase. The results, however, do not exclude a smaller fraction of isoform with high sensitivity to ouabain. The final demonstration will require hybridization experiments of mRNA with probes specific for each of the known isoforms of Na,K-ATPase. The value of K_I is identical to K_I previously reported in the distal colon of adult rats [15]. The values of K_D are similar to the K_D identified in the membrane-rich fraction of colonocytes and jejunal enterocytes from adult rats [19, 20]. In the colon and small intestine of the rabbit, which is more sensitive to ouabain than the rat, K_D was found to be lower [31, 32]. The finding that K_D in isolated colonocytes was lower than K_I in intact epithelium and in epithelial homogenate is likely due to the fact that K_D was measured in the presence of vanadate and absence of K^+, whereas K_I was measured in the presence of K^+ and absence of vanadate.

Finally our data demonstrate that the development of Na^+ extrusion was accompanied by an age-dependent decrease in the affinity for Na^+ as indicated by 1.7 times higher value of $K_{0.5}$ in adult rats than in young animals, without any change in the maximal rate of Na^+ transport. However, the basal rate of active Na^+ extrusion via basolateral membrane may be to a large extent determined not only by the developmental changes of $K_{0.5}$ but also by changes in intracellular activity of Na^+. So far the developmental decrease of Na^+ activity was described only in skeletal muscle [39], and it is not sure whether epithelial cells with large transport pool of Na^+ are subjected to similar changes. In addition, the large developmental changes of K^+ uptake in skeletal muscle cannot be accounted for by changes in intracellular Na^+ [22]. The age-dependent increase in the number of Na,K pumps suggests that a similar increase in Na,K-ATPase activity is due to a change in the number of pump sites. The catalytic turnover number is within the range reported for other tissues [19] and very similar to the values of rabbit colon [31] and rabbit collecting tubule [10], which is an epithelium with transport properties similar to those of colon. However, all of these values are much smaller than the value 10,000 per min usually quoted for purified Na,K-ATPase. This suggests that factors within the cell and/or cell membrane may control the turnover. This is consistent with previous findings of higher catalytic turnover number in more purified membrane preparation of intestinal Na,K-ATPase [19]. Using the data from this study, the maximum turnover rate for Na^+ in intact epithelium can be estimated by the following equation:

$$\text{turnover rate} = 3[(I_{sc}^N/F) \cdot N \cdot 60]/\text{Na,K-pump density} \qquad (5)$$

where F and N are Faraday's constant and Avogadro's number. Factor 3 is the consequence of pump stoichiometry $3Na^+/2K^+$. It was demonstrated that in nystatin-treated epithelium I_{sc}^N was only one-third of net Na^+ flux [28] because K^+ can diffuse across the apical membrane through the nystatin channels and contribute to measured I_{sc}^N. If we suppose that the pump density in the young epithelium is 69×10^{12} sites/cm^2 and in that of adults 784×10^{12} sites/cm^2, the maximum turnover rate would be 500 Na^+/site · min in adult and 6400 Na^+/site · min in young rats. These values are within the range reported for other epithelia such as rabbit colon [31] and tadpole and adult frog skin [4, 30], however, much smaller than the turnover rate of purified Na,K-ATPase, 30,000 Na^+/site · min. Hence cellular factors appear to control the turnover of the pump. These factors may include phospholipids, protein kinase C or Ca^{2+} and some intracellular

proteins [44]. Also the comparison of I_{sc}^{N} and activity of Na,K-ATPase converted to the same unit (μmol Na^+/hr · cm^2), which shows that the maximal pumping rate based on enzymatic measurements is four times higher in 10-day-old and more than 10 times higher in adult rats, indicates the possible regulation of turnover by the cell. Colonocytes may have an intracellular pool of Na,K-ATPase which is variable during development as was demonstrated in some other cells [43], or there may be inactive pumps at the surface of the cells [4].

It is possible that the higher pump turnover rate reflects the action of corticosteroids because this turnover is higher in the distal colon of adult rabbits with secondary hyperaldosteronism than in animals with unstimulated adrenal glands [31]. Previous studies from our laboratory have shown that the high Na^+ transport in sucklings is induced by adrenal corticosteroids and that the colonic epithelium of young rats is more sensitive to these hormones than in adulthood [26, 27]. A recent study, however, indicates some differences between the action of corticosteroids in young and adult animals. In the adult rabbit and rat colon, corticosteroids increase the number of Na,K pumps and the Na,K-ATPase activity [3, 6, 20, 31, 41]. Failure to demonstrate a decrease of Na,K-ATPase activity and Na^+-pumping activity and the low density of Na,K pumps in early postnatal life indicate the existence of alternate mechanisms responsible for the regulation of Na^+ extrusion in the developing colon.

In summary, the maximum Na^+-pumping activity of the rat distal colon is very high in early postnatal life even though the density of Na,K pumps and Na,K-ATPase activity are lower than in adulthood. The high rate of basolateral Na^+ extrusion is a result of increased turnover rate of the Na,K pump for Na^+. The study demonstrates that there may be discrepancy between the results obtained in whole cells and studies on isolated membranes as has also been demonstrated by others [24, 31, 35].

The authors are very grateful to Prof. J. Duhm (Universität München), Prof. P. Hahn (University of British Columbia, Vancouver), Drs. P. Hník and J. Zicha (Institute of Physiology, Prague), Prof. O. Koldovský (University of Arizona, Tucson, AZ), and Prof. L.G. Palmer (Cornell University Medical College, New York, NY) for fruitful discussions and support, Dr. J. Vorlíček (Department of Biomathematics, Institute of Physiology, Prague) for performance of nonlinear regression analysis, and Mrs. B. Doležalová for typing the manuscript.

References

1. Akera, T., Cheng, V.K. 1977. A simple method for the determination of affinity and binding site concentration in receptor binding studies. *Biochim. Biophys. Acta* **470:**412–423
2. Bastl, C.P. 1987. Regulation of cation transport by low doses of glucocorticoids in in vivo adrenalectomized colon. *J. Clin. Invest.* **80:**348–356
3. Binder, H.J. 1978. Effect of dexamethasone on electrolyte transport in the large intestine of the rat. *Gastroenterology* **75:**212–217
4. Cala, P.M., Cogswell, N., Mandel, L.J. 1978. Binding of [³H]ouabain to split frog skin. The role of the Na,K-ATPase in the generation of short circuit current. *J. Gen. Physiol.* **71:**347–367
5. Charney, A.N., Donowitz, M. 1978. Functional significance of intestinal Na^+-K^+-ATPase: in vivo ouabain inhibition. *Am. J. Physiol.* **234:**E629–E636
6. Charney, A.N., Wallach, J., Ceccarelli, S., Donowitz, M., Costenbader, C.I. 1981. Effects of spironolactone and amiloride on corticosteroid-induced changes in colonic function. *Am. J. Physiol.* **241:**G300–G305
7. Del Castillo, J.R., Whittembury, G. 1987. Na^+,K^+ and Cl^- transport in isolated small intestinal cells from guinea pig. Evidences for the existence of a second Na^+ pump. *Biochim. Biophys. Acta* **901:**209–216
8. Dobrovič-Jenik, D., Oželgovič, B., Milkovič, S. 1984. Postnatal development of rat kidney plasma membrane Na-K-ATPase. *Biol. Neonate* **46:**115–121
9. Donaldson, J.R., Schnabel, R.B. 1987. Computational experience with confidence regions and confidence intervals for nonlinear least squares. *Technometrics* **29:**67–82
10. El Mernissi, G., Doucet, A. 1984. Quantitation of [³H]ouabain binding and turnover of Na,K-ATPase along the rabbit nephron. *Am. J. Physiol.* **247:**F158–F167
11. Finkel, Y., Aperia, A., Eklöf, A.-C. 1985. Development of colonic fluid and electrolyte transport: Influence of weaning pattern. *J. Pediatr. Gastroenterol. Nutr.* **4:**457–462
12. Frizzell, R.A., Turnheim, K. 1978. Ion transport by rabbit colon: II. Unidirectional sodium influx and the effects of amphotericin B and amiloride. *J. Membrane Biol.* **40:**193–211
13. Garay, R.P., Garrahan, P.J. 1973. The interaction of sodium and potassium with the sodium pump in red cells. *J. Physiol.* **231:**297–325
14. Grasl, M., Krivanek, P., Turnheim, K. 1982. Does tissue ATP content limit active sodium transport across intestinal epithelia in vitro? *Pfluegers Arch.* **395:**257–259
15. Hafkenscheid, J.C.M. 1973. Occurrence and properties of a (Na^+-K^+)-activated ATPase in the mucosa of the rat intestine. *Pfluegers Arch.* **338:**289–294
16. Halevy, J., Boulpaep, E.L., Binder, H.J., Hayslett, J.P. 1987. Aldosterone increases the maximal turnover rate of the sodium pump. *Pfluegers Arch.* **410:**476–480
17. Halm, D.R., Dawson, D.C. 1983. Cation activation of the basolateral sodium-potassium pump in turtle colon. *J. Gen. Physiol.* **82:**315–329
18. Hansen, O. 1984. Interaction of cardiac glycosides with ($Na^+ + K^+$)-activated ATPase. A biochemical link to digitalis-induced inotropy. *Pharmacol. Rev.* **36:**143–163
19. Harms, V., Wright, E.M. 1980. Some characteristics of Na/K-ATPase from rat intestinal basal lateral membranes. *J. Membrane Biol.* **53:**119–127
20. Hayslett, J.P., Myketey, N., Binder, H.J., Aronson, P.S. 1980. Mechanism of increased potassium secretion in potassium loading and sodium deprivation. *Am. J. Physiol.* **239:**F378–F382
21. Jørgensen, P.L., Skou, J.C. 1971. Purification and characterization of ($Na^+ + K^+$)-ATPase I. The influence of detergents on the activity of ($Na^+ - K^+$)-ATPase in preparations from

the outer medulla of rabbit kidney. *Biochim. Biophys. Acta* **233**:366–380
22. Kjeldsen, K., Nørgaard, A., Clausen, T. 1985. Effects of ouabain, age, and K-depletion on K-uptake in rat soleus muscle. *Pfluegers Arch.* **404**:365–373
23. Lowry, D.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275
24. Lytton, J. 1985. Insulin affects the sodium affinity of the rat adipocyte (Na^+, K^+)-ATPase. *J. Biol. Chem.* **260**: 10075–10080
25. Morris, A.P., Gallacher, D.V., Lee, J.A.C. 1986. A large conductance, voltage- and calcium-activated K^+ channels in the basolateral membrane of rat enterocytes. *FEBS Lett.* **206**:87–92
26. Pácha, J., Popp, M., Čapek, K. 1987. Amiloride-sensitive sodium transport of the rat distal colon during early postnatal development. *Pfluegers Arch.* **409**:194–199
27. Pácha J., Popp, M., Čapek, K. 1988. Corticosteroid regulation of Na^+ and K^+ transport in the rat distal colon during postnatal development. *J. Dev. Physiol.* **10**:531–540
28. Palmer, L.G., Speez, N. 1986. Stimulation of apical Na permeability and basolateral Na pump of toad urinary bladder by aldosterone. *Am. J. Physiol.* **250**:F273–F281
29. Powell, D.W. 1986. Ion and water transport in the intestine. *In*: Membrane Transport Processes in Organized System. T.E. Andreoli, J.F. Hoffman, D.D. Fanestil, and S.G. Schultz, editors. pp. 175–212. Plenum Medical Book, New York
30. Robinson, D.H., Mills, J.W. 1987. Ouabain binding in tadpole ventral skin I. Kinetics and effect on intracellular ions. *Am. J. Physiol.* **253**:R402–R409
31. Roden, M., Turnheim, K. 1988. Sodium pump quantity and turnover in rabbit descending colon at different rates of sodium absorption. *Pfluegers Arch.* **413**:181–189
32. Rowling, P.J.E., Sepúlveda, F.V. 1984. The distribution of ($Na^+ + K^+$)-ATPase along the villus crypt-axis in the rabbit small intestine. *Biochim. Biophys. Acta* **771**:35–41
33. Schwarz, S.M., Hostetler, B., Ling, S., Mone, M., Watkins, J.B. 1985. Intestinal membrane lipid composition and fluidity during development in the rat. *Am. J. Physiol.* **248**: G200–G207
34. Silva, P., Charney, A.N., Epstein, F.H. 1975. Potassium adaptation and Na-K-ATPase activity in mucosa of colon. *Am. J. Physiol.* **229**:1576–1579
35. Soltoff, S.P. 1986. ATP and the regulation of renal cell function. *Annu. Rev. Physiol.* **48**:9–31
36. Sweadner, K.J. 1989. Isozymes of the Na^+/K^+-ATPase. *Biochim. Biophys. Acta* **988**:185–220
37. Taussky, H.H., Shorr, E. 1953. A microcolorimetric method for the determination of inorganic phosphorus. *J. Biol. Chem.* **202**:675–681
38. Turnheim, K., Hudson, R.L., Schultz, S.G., 1987. Cell Na^+ activities and transcellular Na^+ absorption by descending colon from normal and Na^+-deprived rabbits. *Pfluegers Arch.* **410**:279–283
39. Ward, K.M., Wareham, A.C. 1983. Changes in intrafibre Na^- and K^+-activity of mouse skeletal muscle during development. *J. Physiol.* **342**:65P–66P
40. Will, P.C., Cortright, R.N., DeLisle, R.C., Douglas, J.G., Hopfer, U. 1985. Regulation of amiloride-sensitive electrogenic sodium transport in the rat colon by steroid hormones. *Am. J. Physiol.* **248**:G124–G132
41. Will, P.C., DeLisle, R.C., Cortright, R.N., Hopfer, U. 1981. Induction of amiloride-sensitive sodium transport in the intestines by adrenal steroids. *Ann. NY Acad. Sci.* **372**:64–78
42. Wills, N.K., Lewis, S.A., Eaton, D.C. 1979. Active and passive properties of rabbit descending colon: A microelectrode and nystatin study. *J. Membrane Biol.* **45**:81–108
43. Wolitzky, B.A., Fambrough, D.M. 1986. Regulation of the ($Na^+ + K^+$)-ATPase in cultured chick skeletal muscle. *J. Biol. Chem.* **261**:9990–9999
44. Yingst, D.R. 1988. Modulation of the Na,K-ATPase by Ca and intracellular proteins. *Annu. Rev. Physiol.* **50**:291–303

Received 29 August 1990