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Rabbit Distal Colon Epithelium: II. Characterization of (Na^+,K^+,Cl^-)-Cotransport and [^{3}H]-Bumetanide Binding

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Summary. Loop diuretic-sensitive (Na^+,K^+,Cl^-)-cotransport activity was found to be present in basolateral membrane vesicles of surface and crypt cells of rabbit distal colon epithelium. The presence of gradients of all three ions was essential for optimal transport activity. (Na^+,K^+) gradient-driven ^{36}Cl fluxes were half-maximally inhibited by 0.14 μM bumetanide and 44 μM furosemide. While ^{86}Rb uptake rates showed hyperbolic dependencies on Na^+ and K^+ concentrations with Hill coefficients of 0.8 and 0.9, respectively, uptakes were sigmoidally related to the Cl^- concentration, Hill coefficient 1.8, indicating a 1 Na^+ : 1 K^+ : 2 Cl^- stoichiometry of ion transport.

The interaction of putative (Na^+,K^+,Cl^-)-cotransport proteins with loop diuretics was studied from equilibrium-binding experiments using [^{3}H]-bumetanide. The requirement for the simultaneous presence of Na^+,K^+, and Cl^-, saturability, reversibility, and specificity for diuretics suggest specific binding to the (Na^+,K^+,Cl^-)-cotransporter. [^{3}H]-bumetanide recognizes a minimum of two classes of diuretic receptor sites, high-affinity (K_{D_1} = 0.13 μM; B_{max_1} = 6.4 pmol/mg of protein) and low-affinity (K_{D_2} = 34 μM; B_{max_2} = 153 pmol/mg of protein) sites. The specific binding to the high-affinity receptor was found to be linearly competitive with Cl^- (K_i = 60 mM), whereas low-affinity sites seem to be unaffected by Cl^-. We have shown that only high-affinity [^{3}H]-bumetanide binding correlates with transport inhibition raising questions on the physiological significance of diuretic receptor site heterogeneity observed in rabbit distal colon epithelium.

Key Words (Na^+,K^+,Cl^-)-cotransport · ^{3}H-bumetanide binding · rabbit distal colon · basolateral membrane · secretory diarrhea

Introduction

The (Na^+,K^+,Cl^-)-cotransport system has been implicated in transepithelial chloride fluxes and may also play an important role in cell volume regulation after exposure of cells to hypertonic media [for recent reviews, *see* 7, 10, 11, 27]. The stoichiometry of cotransport is generally thought to be 1 Na^+: 1 K^+: 2 Cl^-, i.e., the system is electroneutral [10, 38, 42]. Another common feature of (Na^+,K^+,Cl^-)-cotransport in various systems is that it is effectively blocked by loop diuretics in a rank order of potency of benzmetanide > bumetanide > piretanide > furosemide [36, 37, 39]. Recently, it has been possible to correlate specific binding of radiolabeled diuretics with inhibition of (Na^+,K^+,Cl^-)-cotransport activity [16, 24]. Interestingly, in MDCK cells [12] heterogeneity in [^{3}H] piretanide receptor sites has been observed correlating high- and low-affinity sites with the absence and presence of cotransport activity.

In rabbit distal colon epithelium, absorptive and secretory processes for chloride have been characterized by biophysical methods [reviewed in 9]. In surface cells, chloride is absorbed by an electroneutral process presumably involving apical Cl^-/HCO_3^- exchange and an electroneutral downhill exit at the basolateral membrane. Crypt cells are thought to be responsible for cyclic AMP-induced electrogenic chloride secretion implying conductive apical chloride exit and electrically neutral chloride entry at the basolateral cell domain. It is not clear, at present, whether the basolateral process occurs through a coupled (Na^+,Cl^-)-cotransport mechanism [21] or whether potassium plays a direct role in facilitated chloride entry, too [40]. This possibility is difficult to establish in intact tissue as K^+ is required at the basolateral membrane for the operation of the (Na^+,K^+)-pump. Thus, inhibition of Cl^- secretion by omitting K^+ in the serosal bath solution, being suggestive of the presence of (Na^+,K^+,Cl^-)-cotransport, could also be attributable to decreased (Na^+,K^+)-pump activity responsible for the maintenance of the transmembrane Na^+ gradient driving the Cl^- influx.

In the present study, we have investigated anion-coupled cation transport in basolateral membrane vesicles of rabbit distal colon epithelium. It is demonstrated that the (Na^+,K^+,Cl^-)-cotransport

system exists in surface and crypt epithelial cells and that transport properties and sensitivity to loop diuretics resemble the known features of other mammalian cells [10]. [^{3}H]-bumetanide equilibrium binding recognizes high- and low-affinity diuretic receptors in these membranes, the high-affinity sites being correlated with transport inhibition.

Materials and Methods

Materials

$^{86}Rb^+$ (0.5–35 Ci/g; used within 0.5 times its half life (18.5 days) and ^{36}Cl (1–15 mCi/g) were purchased from New England Nuclear as HCL stock solutions; shifts of pH in the assay media by addition of aliquots of the stock were compensated by appropriate buffer. Potassium [^{14}C] thiocyanate (58 mCi/mmol) was from Amersham; stock solutions were kept in ethanol. [^{3}H]-bumetanide and unlabeled bumetanide were gifts of E. Hoffman, Institute of Biological Chemistry A, August Krogh Institute, Copenhagen, Denmark; the specific radioactivity of [^{3}H]-bumetanide (56.3 mCi/mmol) was estimated fluorimetrically [25] by reference to a calibration curve of unlabeled bumetanide. Furosemide, SITS, amiloride and CCCP were obtained from Sigma. Unlabeled bumetanide and furosemide were prepared freshly as a 20-mM stock solution in 0.4 M imidazol, and lowering the pH slowly to 7.5 with acetic acid. Stock solutions (5 mM) of CCCP were kept in ethanol. Hyaluronidase (EC 3.2.1.35; ≈1000 U/mg) was purchased from Boehringer (Mannheim, FRG). All other chemicals were obtained from commercial sources and were of the highest purity available. The electrolyte concentration of salt stock solutions was controlled by flame photometry.

Preparation of Plasma Membrane Vesicles

Basolateral plasma membrane vesicles of surface (BLMS) and crypt (BLMC) cells of rabbit distal colon epithelium were isolated as described in the preceding article [47] and equilibrated in 200 mM mannitol, 50 mM imidazole-acetate, pH 7.4. Batches of vesicles were prepared from several animals and were quick-frozen in liquid nitrogen and kept at −80°C for experiments the following week. Basolateral plasma membrane vesicles of surface cells were also prepared by use of isolated dispersed cells as a source of material instead of scrapings used in the aforementioned standard preparation procedure. Unfortunately, the yield of viable surface cells from rabbit distal colon epithelium was very low (<5% on the basis of trypan blue exclusion) using a previously published chelation method [15] making this cell preparation less suitable as a source for isolation of tight membrane vesicles. However, we could improve the yield to ≈60% viable cells. Segments of distal rabbit colon were cleaned carefully, everted and placed quickly on plexiglass rods mounted on Vibro mixers (type E, Chemap AG, Mönnedorf, Switzerland). Surface epithelial cells were dissociated by vibration (80% of maximum speed) at 37°C for 30 min in the presence of hyaluronidase (final concentration 0.9 mg/ml; ≈900 IU/ml) added to a medium of 120 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 15 mM glucose, 1 mM dithiothreitol, 10 mM EGTA, 10 mM HEPES-NaOH, pH 7.4, and gassed continuously with 95% O_2/5% CO_2. Cells were collected by centrifugation at 200 × g for 5 min, homogenized, and membrane vesicles were prepared by the standard preparative procedure described in the preceding article [47].

Isotopic Flux Measurements

Thawed (37°C) aliquots of membrane vesicles were equilibrated on ice for 1 hr in 200 mM mannitol, 10 μM CCCP, 50 mM imidazole-acetate, pH 7.4, and any other addition, as indicated. CCCP allows free H^+ permeation [22] and short-circuits electrical gradients, possibly induced by the ion gradients employed. The high concentration of permeable buffer ions should prevent build-up of H^+ gradients [32]. ^{36}Cl and ^{86}Rb transport studies were carried out at 37°C in predesignated media as indicated in the figure legends. Routinely, samples were temperature equilibrated at 37°C for 5 min before starting the uptake reaction by sixfold dilution of membrane vesicles into the uptake medium (final volume 60 to 300 μl). Transport was terminated at appropriate time points by withdrawing 50-μl aliquots of the assay mixture and diluting into 1 ml ice-cold stop solution of either 120 mM N-methyl-D-glucamine sulfate, 0.1 mM furosemide, 50 mM imidazole-acetate, pH 7.4, used in ^{36}Cl transport studies or 150 mM K_2SO_4, 0.1 mM furosemide, 50 mM imidazole-acetate, pH 7.4, used in ^{86}Rb transport studies. Alternatively, in initial rate studies (2–12 sec), 40 μl of uptake medium was placed at the bottom of a tube and 10 μl of the membrane suspension on the tube wall just above the radioactive uptake medium. Uptake was initiated by rapidly mixing the two aliquots using a Vortex. The uptake reaction was quenched by rapid addition of 1 ml ice-cold stop solution directly to the assay mixture using a semiautomatic device similar to that of Kessler, Tannenbaum and Tannenbaum [26]. Membranes were collected by vacuum (Millipore) filtration of 0.9 ml aliquots of the diluted membrane suspension on prewetted (with stop solution) 0.45 μm mixed cellulose ester ME 25 filter (Schleicher & Schuell Gmbh., Dassel, FRG) and rinsed with an additional 2 × 2.5 ml of ice-cold stop solution. Filters, typically containing ≈50 μg of protein, were placed in glass vials containing 8 ml Ready-Solve HP scintillation fluid (Beckman, Fullerton, CA) and were counted for radioactivity by liquid scintillation spectrometry. The entire stopping and washing procedure took 15–25 sec. The loss of radioactivity within that time was ≤5% as determined from pilot studies designed as described by Turner, George and Baum [45]. All values are corrected for nonspecific trapping of isotope by the membranes and filtered by subtracting "zero time uptakes" (typically ≤5 an ≤15% of 90-min equilibrium with BLMS and BLMC, respectively) obtained by mixing stop solution and vesicles before the addition of the uptake medium, then filtering and washing as usual.

[^{3}H] Bumetanide Binding

For equilibrium-binding studies, membranes were incubated at 0.62 (BLMS) and 1.25 (BLMC) mg protein/ml in the presence of increasing (0.03–15 μM) concentrations of [^{3}H]-bumetanide for 1 hr at 22°C in 20 mM NaCl, 10 mM K_2SO_4, 50 mM imidazole-acetate, pH 7.4. Aliquots were diluted 25-fold in ice-cold 50 mM imidazol-acetate, pH 7.2. Membranes were collected by vacuum filtration technique and counted for radioactivity as described above. In competition experiments, membranes were incubated in the same medium mentioned above for 1 hr at 22°C in the presence of 1 μM free [^{3}H]-bumetanide and of various concentrations of unlabeled bumetanide and furosemide. Specific [^{3}H] bumetanide binding was assessed by subtraction of the nonspecific

Table 1. Comparison of (Na^+,K^+)-stimulated ^{36}Cl uptake in the BLMS and BLMC fraction

Source of material	(Na^+,K^+)-stimulated ^{36}Cl uptake (nmol/mg · 45 sec)	
	Bumetanide sensitive	Bumetanide insensitive
Scrapings		
BLMS	2.1 ± 1.3 (5)	5.4 ± 1.2 (5)
BLMC	0.55 ± 0.2 (5)	0.65 ± 0.2 (5)
BLMS/BLMC	3.8	9
Surface cells		
BLMS	2.4 (2)	5.3 (2)

$^{36}Cl^-$ uptakes were performed for 45 sec as described in the legend to Fig. 1 and in Materials and Methods. Data are expressed as mean ± SD; *n* determinations from different preparations are shown in parentheses. BLMS/BLMC indicates activity ratio.

binding component (in the presence of ≥100-fold excess of unlabeled bumetanide) from the total binding (in the absence of unlabeled bumetanide). Binding of [^{3}H]-bumetanide to the filter alone was ≤25% of nonspecific binding.

Protein Determinations

Total protein was estimated by Coomasie blue G-250 dye binding (Biorad) with gamma-globulin as standard. Membranes were solubilized in 0.1% Triton-X100.

Data Analysis

Transport-kinetic and equilibrium-binding data were analyzed as indicated, either graphically or by unweighted regression analysis; software programs were generously supplied by Dipl. Ing. W. Wyskowsky (Department of Pharmacology, University Vienna). Significance was determined using the Student's *t* test.

Results

Na^+,K^+ Gradient-Driven ^{36}Cl Transport

The presence of a (Na^+,K^+,Cl^-)-cotransport system in basolateral membrane vesicles of rabbit distal colon epithelium would be suggested by the ability of a concentration gradient of both Na^+ and K^+ to serve as driving force for Cl^- transport. The time course of $^{36}Cl^-$ uptake in vesicles derived from surface (BLMS) and crypt (BLMC) epithelial cells is illustrated in Fig. 1 as a function of transmembrane cation gradients. Indeed, imposition of an outside > inside Na^+ (200 mM) and K^+ (20 mM) gradient led to a marked stimulation of $^{36}Cl^-$ uptake when compared to permeation in the presence of equimolar N-methyl-D-glucamine replacing the cations. This Na^+- and K^+-dependent acceleration of $^{36}Cl^-$ equilibration suggests a flux coupling for Na^+,K^+,Cl^- membrane translocation either directly via cotransport systems and/or indirectly by diffusion potentials induced by the cation gradients employed. However, under the conditions used the uptake of the membrane permeant anion [^{14}C] thiocyanate (1 mM) in BLMS vesicles in the absence (0.93 ± 0.35 nmol [^{14}C] SCN^-/mg · 45 sec, $n = 8$) and presence (0.96 ± 0.11 nmol [^{14}C]SCN^-/mg · 45 sec, $n = 7$) of the Na^+ and K^+ gradient was not significantly ($P = 0.83$) different arguing against indirect coupling of Cl^- influx to diffusion potentials generated by cation-conductive pathways.

Bumetanide, a rather specific inhibitor of (Na^+,K^+,Cl^-)-cotransport in other mammalian cells [3, 37, 39], virtually abolished the Na^+,K^+ stimulation of $^{36}Cl^-$ uptake in both fractions. The background permeation of $^{36}Cl^-$, i.e., the uptake in the absence of Na^+ and K^+ gradients, was unaffected by bumetanide. By 90 min, all uptakes were approaching equilibrium values, which were similar under either experimental condition. Thus, it appears that the imposed ion gradients and effectors exerted their effects on the uptake process rather than on vesicular volume, membrane integrity or binding.

The BLMS fraction exhibited both a faster uptake rate and a larger capacity than BLMC, consistent with the 3.8-fold higher functional space per unit protein in the former fraction (*see* Table 4 in the preceding article [47]). Taken 45-sec uptake rates, the BLMS/BLMC activity ratio was 3.8 for the bumetanide-sensitive and 9 for the bumetanide-insensitive $^{36}Cl^-$ permeation (Table 1), whereas at 90-min equilibrium the BLMS/BLMC ratio of intravesicular trapped $^{36}Cl^-$ averages again 3.9 under either experimental condition (Fig. 1). The relatively higher background permeation in the BLMS fraction reflects a higher intrinsic Cl^- permeability of BLMS vesicles compared to BLMC.

(Na^+,K^+,Cl^-)-cotransport is thought to represent the basolateral functional component of transepithelial Cl^- transport in secretory epithelia [20, 45]. However, in mammalian colon only crypts seem to be involved in electrogenic Cl^- secretion [46] and it is surprising that (Na^+,K^+,Cl^-)-cotransport activity also seems to be present in surface cell-derived basolateral membranes. Therefore, the possibility of contamination of BLMS by crypt cell basolateral membranes was examined. BLMS vesicles were prepared either simultaneously with BLMC vesicles using total mucosal scrapings as a source of biological material, or by fractionation of

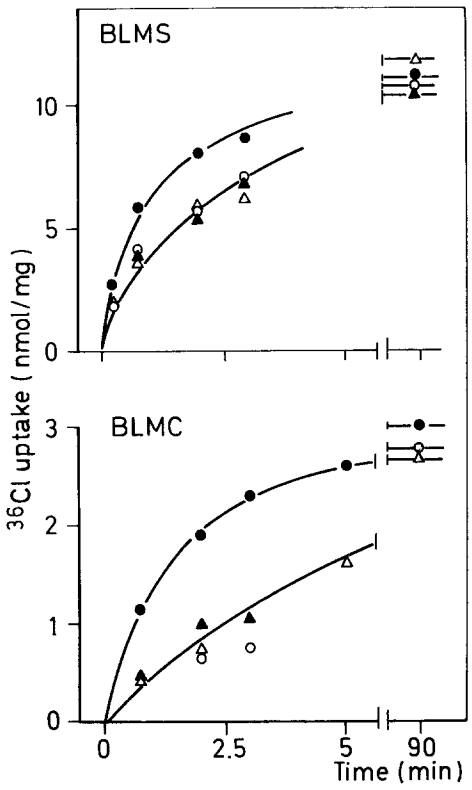

Fig. 1. (Na^+,K^+) gradient-driven $^{36}Cl^-$ uptake in basolateral membrane vesicles of rabbit distal colon epithelium. Vesicles were equilibrated in 200 mM mannitol, 2 mM KCl, 2.5 mM Na_2SO_4, 10 μM CCCP, 50 mM imidazole-acetate, pH 7.4, with and without 0.1 mM bumetanide before diluting sixfold into the uptake medium containing either 100 mM Na_2SO_4 and 10 mM K_2SO_4 (circles) or equimolar N-methyl-D-glucamine SO_4 (triangles) in 50 mM imidazole-acetate, pH 7.4, 0.5 mM $MgSO_4$, 10 μM CCCP, 20 mM $^{36}Cl^-$, final protein 1.2 mg/ml. $^{36}Cl^-$ uptakes in the presence (open symbols) and absence (filled symbols) of 0.1 mM bumetanide proceeded to the indicated time points before being quenched by vacuum filtration as detailed in Materials and Methods. Representative experiments obtained with BLMS (top) and BLMC (bottom) are illustrated

isolated surface epithelial cells. Both preparations were tested for $^{36}Cl^-$ transport properties. The results shown in Table 1 indicate comparable activities in both bumetanide-sensitive and -insensitive ^{36}Cl permeation for either BLMS preparation. Thus, (Na^+,K^+,Cl^-)-cotransport activity in the BLMS fraction is obviously not due to contamination by BLMC vesicles.

A much stronger indication of direct Na^+,K^+,Cl^- flux coupling is suggested by the observation that loop diuretic-sensitive $^{36}Cl^-$ uptake into vesicles is not only dependent on the simultaneous presence of Na^+ and K^+, but also requires transmembrane gradients of both of these ions. As shown in Table 2, furosemide-sensitive $^{36}Cl^-$ uptake

Table 2. Dependence of furosemide-sensitive $^{36}Cl^-$ uptake on the simultaneous presence of Na^+ and K^+ gradients

Condition	Relative $^{36}Cl^-$ uptake	
	BLMS	BLMC
Na^+ gradient, K^+ gradient	100	100
Na^+ gradient, K^+ equilibrium	13	17
Na^+ equilibrium, K^+ gradient	9	11
Na^+ equilibrium, K^+ equilibrium	15	17

Vesicles were preloaded for 4 hr on ice with 200 mM Na^+ and/or 20 mM K^+ (gluconate salts) in a medium containing 10 μM CCCP, 50 mM imidazol-acetate, pH 7.4. Isotonicity was maintained by mannitol. In parallel, samples were preincubated in the absence of cations. $^{36}Cl^-$ uptakes were measured for 45 sec in the presence and absence of 1 mM furosemide. The Na^+ and K^+ concentrations of the uptake media were such that after sixfold dilution of the preloaded vesicles into the assay medium the extravesicular cation concentration either established an outside > inside gradient of 200 mM Na^+ and/or 20 mM K^+ or was equal to the intravesicular concentration. Other details as in Fig. 1. Relative uptake rates (percent of maximum) are the average (SD ≤ 19%) of three to four experiments.

was reduced 80% (BLMC) to 90% (BLMS) when Na^+ and K^+ gradients were eliminated.

The dose-effect relationship of loop diuretics for (Na^+,K^+) gradient-driven $^{36}Cl^-$ transport in BLMS and BLMC vesicles is illustrated in Fig. 2. At the experimental conditions employed, bumetanide and furosemide inhibited the transport system in a dose-dependent manner with half-maximal inhibition (I_{50}) at 0.14 and 44 μM, respectively. For comparison, the half-maximal inhibition of Cl^- secretion in the intact rabbit distal colon epithelium occurs at 50 μM furosemide [21], which is in excellent agreement to the values obtained with the vesicle preparation. Obviously, there is no significant difference between the two membrane fractions with respect to their sensitivity to the inhibitors used. When experimental uptake data are analyzed in terms of the Hill equation as modified by Chou [4], the calculated apparent Hill coefficients of inhibitor interaction at 20 mM extravesicular Cl^- were 0.54 and 0.59 with bumetanide and furosemide, respectively. These data suggest either cooperative effects and/or interaction stoichiometries of $\frac{1}{2}$, indicating that one inhibitor molecule prevents permeation of two chloride ions.

(Na^+,Cl^-) Gradient-Driven $^{86}Rb^+$ Transport

Since loop diuretic-sensitive $^{36}Cl^-$ uptake was dependent on both Na^+ and K^+ gradients, it follows that K^+ uptake should be dependent on Na^+ and Cl^- gradients. Figure 3 and Table 3 demonstrate

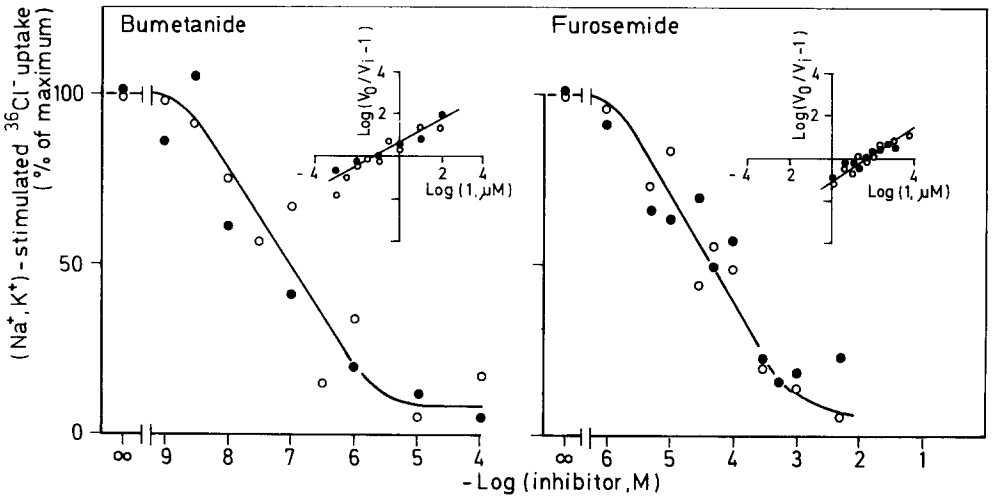

Fig. 2. Inhibition of Na^+,K^+ gradient-driven $^{36}Cl^-$ uptake by loop diuretics. $^{36}Cl^-$ uptakes were performed for 45 sec with BLMS (open circles) and BLMC (filled circles) vesicles in the presence of an outside > inside Na^+ (200 mM outside *vs.* 5 mM inside the vesicle) and K^+ (20 mM outside *vs.* 2 mM inside the vesicle) gradient as described in Fig. 1, except that increasing concentrations of inhibitors were included in both the equilibration and the uptake medium. Fractional uptakes with respect to the uninhibited system (1.83 and 0.51 nmol $^{36}Cl^-$/mg · 45 sec with BLMS and BLMC, respectively) as a function of the inhibitor concentration (log scale) are shown. Insets: replot of the data according to Chou [4]; v_o,v_i, uptake rates in the absence and presence of inhibitor, respectively; I, concentration (μM) of inhibitor. The Hill coefficients of the inhibitor interaction (slopes of the dose-effect lines) are 0.54 and 0.59, and I_{50}, the concentrations required for 50% inhibition (the medium-effect axis intercept, i.e., log $[v_o/v_i - I] = 0$) are 0.14 and 44 μM with bumetanide and furosemide, respectively. The correlation coefficient, by linear regression analysis, was ≥0.912

that this was indeed the case when measuring $^{86}Rb^+$ uptake. Rb^+ was previously found to substitute quantitatively for K^+ [28, 35, 36] in the (Na^+,K^+,Cl^-)-cotransport system. If Cl^- was replaced by gluconate and vice versa Na^+ was replaced by N-methyl-D-glucamine, there was no significant furosemide-sensitive $^{86}Rb^+$ uptake by BLMS and BLMC vesicles. The BLMS/BLMC activity ratios for furosemide-sensitive and -insensitive $^{86}Rb^+$ uptake as well as for the 90-min ^{86}Rb equilibrium values were in close agreement to that obtained with $^{36}Cl^-$ uptakes (*see above*). Also shown in Table 3 is that neither SITS, a widely used inhibitor of anion exchange [3], nor amiloride, the Na^+-binding site competitive inhibitor of Na^+/H^+ exchange [29], altered the response to the NaCl gradient. When furosemide-sensitive ^{86}Rb uptake was measured, it was found to be a pseudolinear function of time over a 12-sec period and of protein up to a concentration of 1.8 mg protein/ml uptake medium (insets in Fig. 3). On this basis, subsequent measurements were made at 10 sec and 1.2 mg of protein/ml assay medium. Under these conditions, total intravesicular ^{86}Rb was ≤15% of the 90-min equilibrium value.

Efforts to further characterize the properties of the basolateral membrane (Na^+,K^+,Cl^-)-cotransport in rabbit distal colon epithelium included an evaluation of its dependence on simultaneously varied Na^+, Cl^- and K^+ concentration gradients shown in Fig. 4. When Cl^--stimulated furosemide-sensitive ^{86}Rb uptake was measured as a function of Na^+ and K^+ concentration, a hyperbolic relationship was obtained with apparent Hill coefficients (n) of 0.8 for Na^+ and 0.9 for K^+. The dependency of Na^+-stimulated ^{86}Rb uptake on the Cl^- concentration gradient was sigmoidal giving an apparent Hill coefficient of 1.8, which is suggestive of a minimum of two Cl^--binding sites. The concentration of Na^+,

Table 3. Effect of ion substitution and ion transport inhibitor on furosemide-sensitive $^{86}Rb^+$ uptake

Incubation medium	Percent uptake	
	BLMS	BLMC
NaCl	100	100
Na gluconate	6	1
N-methyl-D-glucamine Cl	10	7
NaCl + SITS (1 mM)	114	105
NaCl + amiloride (0.1 mM)	98	107

$^{86}Rb^+$ uptakes were measured as described in Fig. 3 in the presence of ion gradients (outside > inside) of either 150 mM NaCl or equimolar Na gluconate or N-methyl-D-glucamine chloride. Values (mean of four experiments, SD ≤ 18%) indicated are the percent of furosemide-sensitive uptake rate in the presence of NaCl (32 and 8 pmol ^{86}Rb/mg · 45 sec, for BLMS and BLMC, respectively).

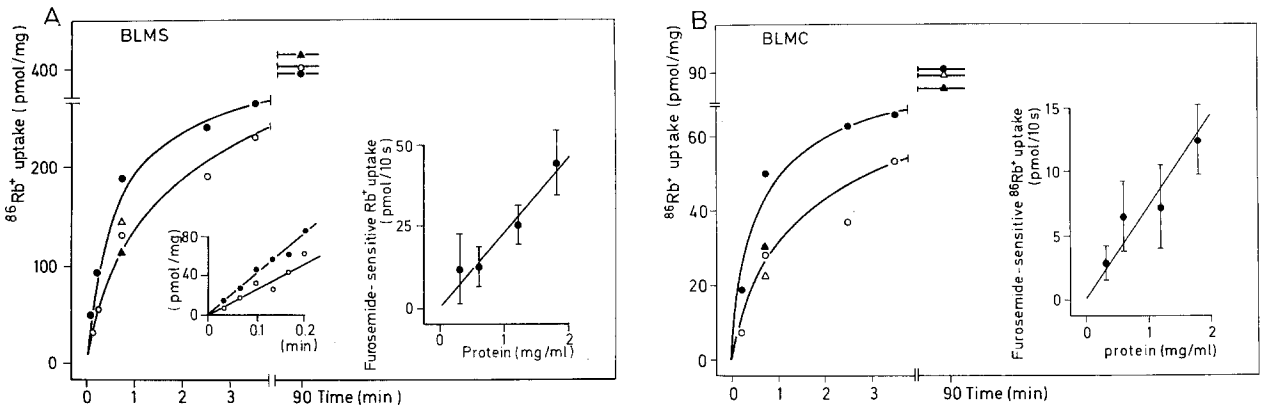

Fig. 3. NaCl gradient-stimulated $^{86}Rb^+$ uptake in basolateral membrane vesicles of rabbit distal colon epithelium. Vesicles were equilibrated in 200 mM mannitol, 10 μM CCCP, 50 mM imidazol-acetate, pH 7.4, with and without 1 mM furosemide before diluting into the assay medium containing (final extravesicular concentration) 150 mM NaCl (circles) or 150 mM Na gluconate (triangles), 0.5 mM $MgSO_4$, 10 μM CCCP, 0.5 mM $^{86}Rb^+$, 50 mM imidazol-acetate, pH 7.4; final protein 0.3 to 1.8 mg/ml. Uptakes were performed in the absence (filled symbols) and presence (open symbols) of 1 mM furosemide and were terminated at the indicated times using the standard vacuum filtration procedure. (*A*) Representative time course of $^{86}Rb^+$ uptake in the BLMS fraction; initial rates are shown in the left-hand inset on an extended time scale. The right-hand inset shows furosemide-sensitive $^{86}Rb^+$ uptake as a function of protein concentration (points are the mean ± SD (error bars) for three to five determinations). (*B*) The equivalent data obtained for the BLMC fraction. Note the different uptake scale

which was half-maximal for stimulation of Cl^--dependent ^{86}Rb uptake, was $K_{0.5}^{Na} = 0.6$ mM in the presence of 20 mM K^+ and 140 mM Cl^-. When the concentration of K^+ was varied and Na^+ and Cl^- were held constant at 140 mM, this value was $K_{0.5}^{K} = 7.6$ mM. The Cl^- concentration required for half-maximal stimulation of ^{86}Rb uptake at 140 mM Na^+ and 20 mM K^+ was $K_{0.5}^{Cl} = 28$ mM. The apparent V_{max} values of furosemide-sensitive ^{86}Rb uptake at infinite concentrations of the varied ions were 365, 361 and 359 pmol/mg · 10 sec, determined from replots of the data from the saturation curves by the method of Eadie-Hofstee in the format of v *vs.* $v/[Na^+]^{0.8}$, v *vs.* $v/[Cl^-]^{1.8}$, and v *vs.* $v/[K^+]^{0.9}$ graphs, respectively. The close agreement in maximal uptake rates under each condition indicates that V_{max} conditions for the two fixed ions were established while varying the concentration of the third ion.

Equilibrium Binding of [^{3}H]-Bumetanide

Bumetanide has been shown to be a useful ligand for the identification of the diuretic-binding site of the (Na^+,K^+,Cl^-)-cotransport system in mammalian cells [17, 24, 25]. Since earlier studies [8, 16] had indicated that all three ions, Na^+, K^+ and Cl^-, are required for optimal [^{3}H]-bumetanide binding, we also examined the appropriate ions as to their effect on [^{3}H]-bumetanide binding. In preliminary equilibrium-binding experiments performed at 1 μM free [^{3}H]-bumetanide, we found that inclusion of 20 mM KCl and 20 mM Na gluconate in the assay medium increased specific [^{3}H]-bumetanide binding by approximately 40%. No significant difference was obtained whether the incubation period was 30 or 60 min, indicating that equilibrium has been attained.

Figure 5 demonstrates the concentration dependence of [^{3}H]-bumetanide binding to the BLMS fraction. Nonspecific binding, as measured in the presence of 1.5 mM unlabeled bumetanide, was a linear function of the free bumetanide concentration up to 15 μM ($r = 0.98$); specific binding represents ≥50% of the total binding. The degree of free radioligand depletion by binding to the membrane fractions was negligible (≤3%) under the experimental conditions used. Replots (inset, Fig. 5) of the specific binding component in the format of a Scatchard graph [43] were nonlinear, suggesting the presence of at least two binding sites for [^{3}H]-bumetanide. The binding parameters, as estimated by nonlinear regression analysis of the data set ($n = 38$) using a two-site model were $K_{D_1} = 0.13$ μM and $B_{max_1} = 6.4$ pmol/mg for the high-affinity binding sites and $K_{D_2} = 34$ μM and $B_{max_2} = 153$ pmol/mg for the low-affinity sites (residual sum of squares 6.4). However, it should be noted that, if the same data were replotted on a semilogarithmic graph according to Klotz [30], it was apparent that the slope of the binding curve has not turned around at an inflection point (*not shown*). Thus, the highest experimental point obtained at 15 μM free [^{3}H]-bumetanide is ≤50% of the total saturation value. Therefore, the total number of [^{3}H]-bumetanide-binding

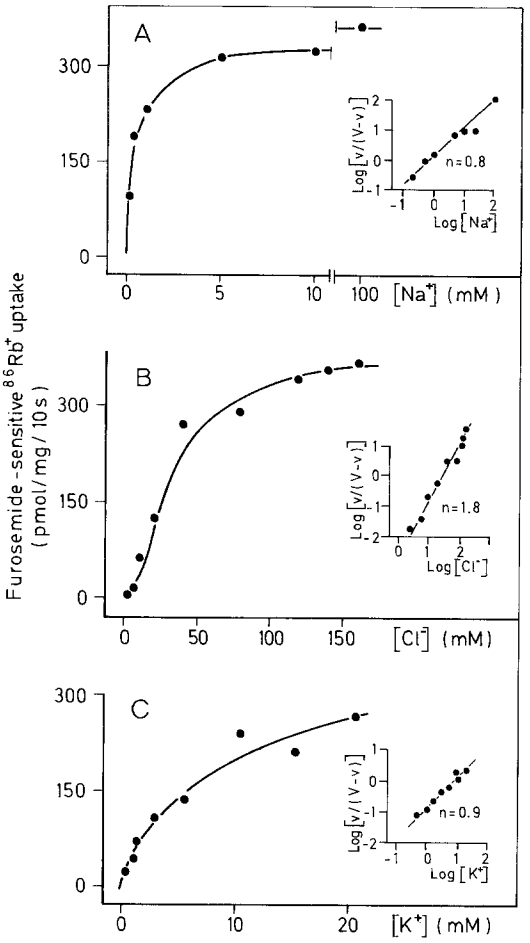

Fig. 4. Furosemide-sensitive $^{86}Rb^+$ uptake as a function of Na^+, Cl^-, and K^+ concentration. ^{86}Rb uptakes were carried out as in Fig. 3 with the BLMS fraction (final protein 1.2 mg/ml) in a medium containing 0.5 mM $MgSO_4$, 10 μM CCCP, 0.5 mM $^{86}Rb^+$, 50 mM imidazole-acetate, pH 7.4, with and without 1 mM furosemide under either of the following conditions: (*A*) 0.2–140 mM Na^+, 20 mM K^+, and 140 mM Cl^-; (*B*) 2–160 mM Cl^-, 20 mM K^+, and 140 mM Na^+; (*C*) 0.5–20 mM K^+, 140 mM Na^+ and 140 mM Cl^-. Isotonicity was maintained while simultaneously varying Na^+, Cl^-, and K^+ concentrations by using either Cl^- and gluconate salts of Na^+ and K^+, N-methyl-D-glucamine chloride or mannitol. Uptakes were terminated after 10 sec using the standard filtration procedure. *Main figure:* Saturation curves of furosemide-sensitive $^{86}Rb^+$ uptake. *Insets:* Replot of the data in a Hill graph with apparent Hill coefficients (n) obtained by linear regression analysis (correlation coefficient ≤ 0.978); v, 10-sec uptake rate, V, apparent maximal uptake rate estimated from v *vs.* $v/[\text{ion}]^n$ plots

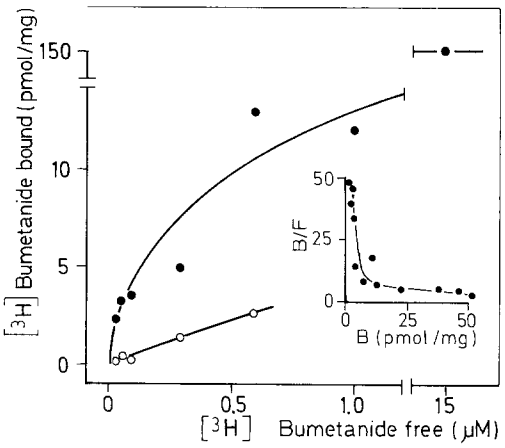

Fig. 5. Equilibrium binding of [^{3}H]-bumetanide to basolateral membranes of rabbit distal colon epithelium. Increasing concentrations of [^{3}H]-bumetanide were incubated with aliquots of the BLMS fraction (0.6 mg of protein/ml) for 1 hr at 22°C in a medium containing 20 mM NaCl, 10 mM K_2SO_4, 50 mM imidazol-acetate, pH 7.4, without (closed circles) or with (open circles) unlabeled bumetanide (1.5 mM). *Inset:* Scatchard plot of saturable binding. B/F = bound/free. Solid curve is computerized best fit for a two-site model

sites cannot be evaluated with sufficient accuracy and the values indicated may only represent the minimum amount of actual binding sites.

Competition equilibrium-binding data, in Fig. 6, show that specific [^{3}H]-bumetanide binding to basolateral membranes of rabbit distal colon epithelium was inhibited progressively by increasing concentrations of unlabeled ligands. At 1 μM free [^{3}H]-bumetanide, half-maximal displacement of site-bound radioactivity was observed at concentrations of 30 and 800 μM of unlabeled bumetanide and furosemide, respectively. Note, however, that high-affinity binding sites coexist with low-affinity sites in these membranes (*see* Fig. 5), the amount of the former being at least one order of magnitude lower. Thus, the competition curves shown in Fig. 6 represent the additive effect of displacement at both low- and high-affinity binding sites. On comparing competition binding data with cotransport inhibition, it can be seen that half-maximal radioligand competition occurs at concentrations one to two orders of magnitude higher than half-maximal transport inhibition (*see* Fig. 2). One possible explanation for this inconsistency would be that only part of the bumetanide receptor sites are identical to the inhibitory sites on the active (Na^+,K^+,Cl^-)-cotransporter.

Recently, saturable [^{3}H]-bumetanide binding has been shown to be inhibited by high Cl^- concentrations [8, 16, 19]. We also noted that Cl at concentrations of 10 to 300 mM has an inhibitory effect on saturable [^{3}H]-bumetanide binding to basolateral membranes of rabbit distal colon epithelial cells. Figure 7 shows the equilibrium-binding data obtained at 0.3, 1 and 10 μM free [^{3}H]-bumetanide as a function of Cl^- concentration plotted in the format of a Dixon graph. The pattern obtained indicates simple competitive inhibition (K_i = 60 mM); i.e., a family of straight convergent lines intersecting

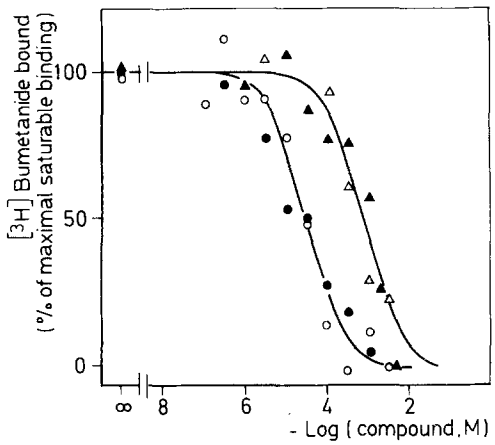

Fig. 6. Effects of loop diuretics on saturable [^{3}H]-bumetanide binding. Specific binding of [^{3}H]-bumetanide (1 μM) was measured after a 1 hr incubation with BLMS (0.6 mg protein/ml) and BLMC (1.3 mg protein/ml) at 22°C in the presence of 20 mM NaCl, 10 mM K_2SO_4, 50 mM imidazol-acetate, pH 7.4, and increasing concentrations of unlabeled bumetanide (circles) and furosemide (triangles). Nonspecific binding was determined in a parallel incubation performed with 1.5 mM bumetanide and subtracted from the data. [^{3}H]-bumetanide-specific binding (100%) to BLMS (open symbols) and BLMC (filled symbols) represents 9.2 and 6.5 pmol/mg of protein, respectively.

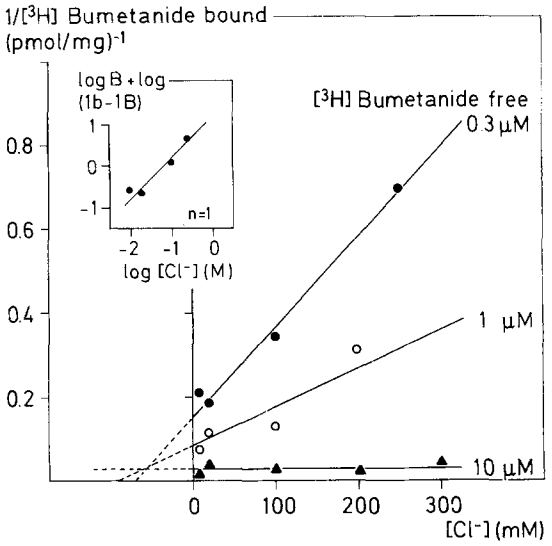

Fig. 7. Inhibition of specific [^{3}H] bumetanide binding by Cl^-. The final binding medium contained aliquots of the BLMS fraction (0.6 mg protein/ml), [^{3}H] bumetanide at a concentration of 0.3 μM (●), 1 μM (○), and 10 μM (▲), 20 mM + K^+, 300 mM Na^+, the indicated concentrations of Cl^-, 50 mM imidazole-acetate, pH 7.4. Cations were added as Cl^- or gluconate salts. Equilibrium binding was performed for 1 hr at 22°C in the absence (total binding and presence (nonspecific binding)) of 1 mM unlabeled bumetanide the difference being the specific [^{3}H] bumetanide-binding component. The inhibition constant for Cl^-, K_i = 60 mM, was determined from a Dixon-plot; lines were drawn by eye. Inset: data obtained at 0.3 μM [^{3}H]-bumetanide were replotted according to a Hill-type equation [38]; *B*, apparent maximal specific binding; *b*, specific binding at the indicated concentrations of Cl^-; *n*, Hill coefficient of Cl^- interaction with the bumetanide-binding site

above the [Cl^-] axis with slope replots, *not shown,* passing through the origin [43]. The graphical representation of the equilibrium-binding data obtained at 0.3 μM free [^{3}H]-bumetanide in a modified [33] Hill plot illustrates the characteristic feature of a single-site interaction with unity Hill coefficient and a constant slope of the graph shape (*see* inset, Fig. 7). At 10 μM free [^{3}H-bumetanide an apparently horizontal line was obtained in the Dixon plot, indicating that Cl^- has no significant competing effect in the presence of high radioligand concentrations. However, 10 μM of the radiolabel would be expected to be saturating for the high-affinity bumetanide receptor (K_{D_1} = 0.13 μM), but subsaturating for the low-affinity receptors (K_{D_2} = 34 μM). Thus, if not only the high-affinity but also the low-affinity bumetanide-binding sites are sensitive to Cl^-, we would expect some inhibitory effects at least at high Cl^- concentrations.

The identification of two bumetanide-binding sites (Fig. 5) on one hand, and the apparently single-site inhibition kinetic in (Na^+,K^+) gradient-stimulated ^{36}Cl transport (Fig. 2), on the other hand, suggests that only one of the two diuretic-binding sites seems to be involved in cotransport inhibition. Indeed, the bumetanide concentration producing half-maximal transport inhibition (I_{50} = 0.14 μM) are essentially identical to those producing half-maximal saturation of the high-affinity bumetanide receptor sites (K_{D_1} = 0.13 μM), but they are two orders of magnitude lower than those half-saturating the low-affinity sites (K_{D_2} = 34 μM). This correlation is illustrated even more clearly in Fig. 8 by the closed circles, where high-affinity bumetanide-binding sites were titrated, compared with open circles where cotransport was inhibited by increasing concentrations of bumetanide. Again, fractional inhibition of cotransport activity parallels fractional occupation of the high-affinity bumetanide-binding site. This supports the conclusion that diuretic binding to the high-affinity receptor site seems to be responsible for inhibition of (Na^+,K^+,Cl^-)-cotransport activity in basolateral membrane vesicles of rabbit distal colon epithelial cells.

Discussion

The present study provides strong evidence that (Na^+,K^+,Cl^-)-cotransport activity is present in ba-

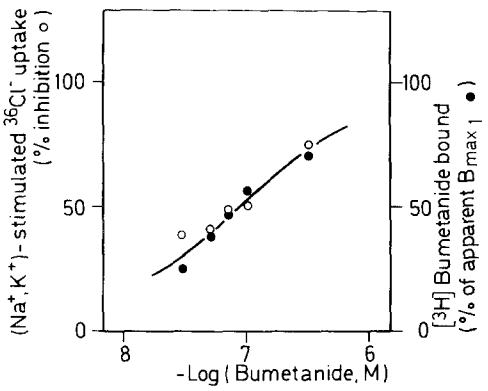

Fig. 8. Correlation between inhibition of (Na^+,K^+)-stimulated ^{36}Cl uptake and occupation of bumetanide high-affinity sites. Fractional inhibition of (Na^+,K^+) stimulated ^{36}Cl uptake (left-hand ordinate; open circles) parallels fractional occupation of the bumetanide high-affinity site (right-hand ordinate; filled circles) labeled to 24 to 72% of maximal binding. Experimental data were obtained with the BLMS fraction

solateral membranes of rabbit distal colon epithelium. This contention is supported by the characteristic ion interdependence and selectivity of loop diuretic-sensitive ^{36}Cl and ^{86}Rb transport in isolated plasma membrane vesicles. As would be predicted for this transport pathway, the stoichiometry of the coupled ion fluxes is 1 Na^+ : 1 K^+ : 2 Cl^-, based on the following indirect evidence: (*i*) loop diuretic-sensitive flux of $^{36}Cl^-$ or $^{86}Rb^+$ (K^+) occurs only in the presence of the other two complementary ions; (*ii*) the apparent Hill coefficients for specific ion gradient-stimulated ^{86}Rb fluxes are 0.8 for Na^+, 0.9 for K^+ and 1.8 for Cl^-; (*iii*) one molecule of a loop diureticum seemingly prevents permeation of two Cl^- ions, as indicated by the approximately half-unity interaction coefficient observed with bumetanide and furosemide in (Na^+,K^+) gradient-stimulated ^{36}Cl fluxes; (*iv*) in kinetic terms, the ratio of apparent V_{max} values of ^{36}Cl transport, $V_{max}^{app} \approx 940$ pmol ^{36}Cl/mg · 10 sec, to that of ^{86}Rb transport, $V_{max}^{app} \approx 360$ pmol $^{36}Rb^+$/mg · 10 sec, averages 2.6. Taking into account the inaccuracies in estimating the V_{max} values for loop diuretic-sensitive ^{36}Cl uptake, this supports a 2 : 1 stoichiometry between chloride and potassium flux; (*v*) loop diuretic-sensitive ^{36}Cl fluxes were not only dependent on the simultaneous presence of Na^+ and K^+, but require transmembrane gradients of these ions (Table 2). This observation strongly supports energetic activation of the transport system, i.e., actual flux coupling of all three ions [44].

The half-maximal stimulating concentrations of (Na^+,K^+,Cl^-)-cotransport for each ion in the presence of saturating concentrations of the other two ions were 0.6, 7.6 and 28 mM for Na^+, K^+ and Cl^-, respectively. These values are comparable to those found in membrane vesicles of other mammalian cells [1, 31, 45]. Notably in the present context, these values for K^+ and Cl^- obtained with basolateral membrane vesicles are in excellent agreement with the corresponding values evaluated in the intact epithelium, 8 mM for K^+ [40] and 20 mM for Cl^- [21], the half-saturation constants for Na^+ are quite different: 0.6 mM in the vesicle preparation *vs.* 15 mM in the intact colon epithelium [21]. The (Na^+,K^+,Cl^-)-cotransport system in cortical thick ascending limb of rabbit kidney has an equally high affinity for Na^+ as we report here for rabbit colon [13]. In addition, recent investigations indicate that K^+ may also bind with low affinity to the Na^+ site of the cotransport system [8].

A striking feature of the present study is that (Na^+,K^+,Cl^-)-cotransport activity seems to be present in basolateral membranes of both surface and crypt cells regardless of their proposed different physiological role in electrolyte transport as an absorptive and secretory system, respectively [46]. Indeed, the cotransport system is thought to be functionally reversible [42], i.e., the direction of the net ion flux is determined by the magnitude of the transmembrane gradients of the three transported ionic species. In crypt cells, it may participate in electrogenic chloride secretion with inwardly directed polarity, as generally proposed for Cl^--secreting epithelia [*see* 7, 42]. In surface cells, Cl^- is absorbed by an electroneutral process [9], which probably involves Cl^-/HCO_3^- exchange at the apical membrane and an electroneutral facilitated downhill transport at the basolateral cell domain, and (Na^+,K^+,Cl^-)-cotransport is a potential mechanism for Cl^- efflux. Furthermore, it is tempting to speculate that this transport system, also plays a role in volume regulation of rabbit distal colon epithelial cells as described for other mammalian cells [10, 11, 23]. However, when normalized to protein, the loop diuretic-sensitive ^{36}Cl and ^{86}Rb transport activity was approximately 3.9-fold greater in the surface cell-derived membrane fraction than in crypt cell membranes, in the same proportion as other vesicle characteristics (e.g., functional space, lipid content, etc.; *see* preceding article [47]). Hence, at least in a first approximation, a similar density of cotransport entities in either membrane fraction may be assumed.

[^{3}H]-bumetanide-binding experiments provided additional information on the molecular properties of the cotransporter in the rabbit distal colon epithelium. Equilibrium binding has been shown to fulfill the criteria predicted for binding to the cotrans-

porter [8], including the requirement for the simultaneous presence of Na^+, K^+, and Cl^- in the incubation medium, saturability, reversibility and specificity for diuretics inhibiting the (Na^+,K^+,Cl^-)-cotransport activity. Unexpectedly, Scatchard plots were curvilinear with upward concavity suggesting the presence of at least two bumetanide-binding sites (K_D = 0.13 and 34 μM), the number of high-affinity sites being over an order of magnitude less than the number of low-affinity sites. The key feature, however, was that only binding to the high-affininty sites parallels transport inhibition. Interestingly, this contrasts to the situation in MDCK cells, an epithelial cell line derived from dog kidney. Again, two classes of diuretic receptors were found, but the low-affinity sites have been shown to be responsible for inhibition of (Na^+,K^+,Cl^-)-cotransport activity [12]. Notably also, high- and low-affinity receptor sites seem to be independently regulated [12]. On the other hand, in red blood cells [16] transport inhibition and saturable binding were half-maximal at $\approx 10^{-7}$ M [^{3}H] bumetanide, which is comparable to the high-affinity bumetanide receptor we found in distal colon epithelium. It will be interesting in the future to evaluate the physiological significance of the heterogeneity in diuretic receptor sites, which correlate differently with cotransport activity.

Assuming 6.4 pmol/mg maximal high-affinity bumetanide binding, (Fig. 5) and if the molecular weight of the cotransport protein were 150 kDa [18], the functionally active cotransporter would comprise only $\approx$0.1% of the total protein of the surface cell-derived membrane fraction. Given a 34-fold purification of the membranes and a protein content of 1.9×10^{-7} mg per single cell (*see* preceding article [47]), we estimate that (Na^+,K^+Cl)-cotransport is represented in rabbit distal colon by a minimum of 21,000 copies per single cell. This is twice the value reported in ferret red cells [34], over an order of magnitude higher than in duck red cells [19], but two orders of magnitude lower than in Ehrlich ascites cells [24].

Assuming, that cotransport activity is inhibited by binding of a single diuretic molecule to the high-affinity bumetanide-binding receptor, a turnover number of 6 sec^{-1} at 37°C for potassium ions can be calculated from the total amount of high-affinity sites (*cf.* Fig. 5) and the maximal loop diuretic-sensitive $^{86}Rb^+$ flux (*cf.* Fig. 4). Although low, this value is within the range of that observed in Ehrlich ascites cells [24], but over one to two orders of magnitude lower than in MDCK [12, 41] and duck red [19] cells.

The existence of two distinct classes of bumetanide-binding sites differing in affinity and capacity as well as in their biological response is of particular interest in view of the possibility that they could represent different functional states of the (Na^+,K^+,Cl^-)-cotransporter [2]: an active-state, high-affinity system actually involved in ion transport and an inactive-state, low-affinity system representing a "physiologically silent cotransporter reserve." If so, alternate conversion of the two systems could represent a short-term kinetic regulation mechanism, which may play a role in transepithelial ion transport and cell volume regulation. Recently, it has been shown in duck red cells [19] that conditions which stimulate (Na^+,K^+,Cl^-)-cotransport activity indeed promote a parallel increase in specific [^{3}H] bumetanide binding. Interestingly, in dog kidney [18] photolabeling experiments have shown that part of the membranes containing low-affinity loop diuretic-binding protein ($\approx$50 kDa) differ in equilibrium density from membranes containing high-affinity receptors ($\approx$150 kDa). It will also be of interest to evaluate whether low-affinity diuretic-receptor sites are associated with subcellular membranes (e.g., Golgi membranes, endoplasmic reticulum) contributing to the background contamination in our basolateral membrane fraction (*see* preceding article [47]). As an alternative explanation, the low-affinity bumetanide-receptor sites might represent cotransport proteins altered during the membrane isolation procedure or other loop diuretic-sensitive transport systems with less sensitivity to these agents than the (Na^+,K^+,Cl^-)-cotransport system. There is evidence that Cl^-/HCO_3^- exchange seems to be present in rabbit distal colon epithelium, at least in apical membranes of surface cells [9], and bumetanide, in the 100-μM range, is known to inhibit anion exchange proteins [14]. Furthermore, (K^+,Cl^-)-cotransport systems are also sensitive to loop diuretics, one to two orders of magnitude less than (Na^+,K^+,Cl^-)-cotransport [6]. However, we could not find conclusive evidence for the presence of (K^+,Cl^-)-cotransport in the isolated membrane fractions. Finally, it must be stressed that allosteric interactions could yield similar curvilinear Scatchard plots [5]. It seems unlikely, however, that this is an important consideration with regard to the results reported herein since high- and low-affinity binding sites could be clearly ascribed to the presence and absence of cotransport activity.

Regardless of the unknown functional significance of the low-affinity bumetanide receptor, both transport and binding studies suggest the existence of a loop diuretic-sensitive (Na^+,K^+,Cl^-)-cotransport system in basolateral membranes of both the absorptive and secretory system of the rabbit distal colon epithelium. Future research will have to be devoted to the physiological significance of diuretic

receptor site heterogeneity. The bidirectional salt transport in colon is complicated by the fact that multiple systems are involved in transport of anyone ion and the fluxes of these ions may be coupled variably to the fluxes of others and modulated by different mechanisms of regulation. These studies indicate that vesicles of crypt- and surface cell-derived membranes may help to shed light on the molecular mechanisms underlying these complex functions and will enable us to obtain a better understanding by which plasma membranes become functionally specialized during differentiation from a secretory to an absorptive epithelium.

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