Conductive pathways for chloride and oxalate in rabbit ileal brush-border membrane vesicles

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Conductive pathways for chloride and oxalate in rabbit ileal brush-border membrane vesicles. Am. J. Physiol. 275 (Cell Physiol. 44): C748–C757, 1998.—To evaluate the possibility that an apical membrane conductive pathway for oxalate is present in the rabbit distal ileum, we studied oxalate ([14C]oxalate) and chloride ([36Cl]) uptake into brush-border membrane vesicles enriched 15- to 18-fold in sucrase activity. Voltage-sensitive pathways for oxalate and chloride were identified by the stimulation of uptake provided by an inwardly directed potassium diffusion potential in the presence of valinomycin. Additionally, outwardly directed oxalate (or chloride) gradients stimulated [14C]oxalate (or [36Cl]) uptake to a greater degree in the absence of valinomycin (when intracellular and extracellular potassium are equal) than in the presence of valinomycin. Voltage-dependent anion uptake was poorly saturable: apparent affinity constants were 141 ± 17 and 126 ± 8 mM for chloride and oxalate, respectively. Activation energies for the voltage-dependent uptake processes were low: 4.7 and 6.3 kcal/mol for chloride and oxalate, respectively. Sensitivity profiles of voltage-dependent chloride and oxalate uptake to anion transport inhibitors were similar. We conclude that an anion conductance is present in the apical membranes of ileal enterocytes and that this conductance is a candidate pathway for oxalate efflux from the enterocyte during transepithelial oxalate secretion.

The purpose of the present study was to test one aspect of this hypothesis, namely, that a conductive pathway for oxalate (and chloride) exists in brush-border membrane vesicle (BBMV) fractions prepared from the rabbit distal ileum. Using this preparation, we found voltage-sensitive oxalate and chloride uptake pathways that are poorly saturated, have low activation energies (E_A), and are inhibited by several putative chloride channel blockers. These results suggest that an apical membrane conductive pathway for the oxalate anion could contribute to cAMP-stimulated oxalate secretion observed in this and other intestinal segments. Whether this oxalate conductance is the same as that for chloride has not been completely resolved.

MATERIALS AND METHODS
Preparation of Membrane Vesicles

Ileal BBMVs were prepared from fresh or frozen mucosal scrapings from New Zealand White rabbits of either gender after an overdose of pentobarbital sodium or decapitation. Mucosal scrapings were snap frozen in liquid nitrogen and could be stored for at least 6 mo without apparent deterioration of the anion transport pathways considered here. A double magnesium-EGTA differential centrifugation method (28) was employed to isolate a membrane vesicle fraction enriched in brush-border membrane marker enzymes (see below). Briefly, ileal scrapings from up to 60 cm of mucosa were homogenized in a buffer (60 mM mannitol, 5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, and 15 mM HEPES-Tris to pH 7.4) with a Waring blender at high speed for 3 min at 4°C. MgCl2 was added to this initial whole homogenate to a final concentration of 12 mM, and the suspension was gently stirred for 10 min in an ice-cold water bath. Aggregated membranes and organellar structures were removed by low-speed centrifugation (1,800 g_max, 15 min), and the supernatant was collected and centrifuged at 20,000 g_max for 30 min. The resultant pellet was resuspended in ice-cold homogenization buffer and homogenized with 10 strokes in a glass-Teflon homogenizer, and the magnesium-EGTA precipitation-differential centrifugation steps described above were repeated. The pellet (P4) was typically resuspended in the desired transport loading buffer, homogenized again with 10 strokes in a glass-Teflon homogenizer, and pelleted by centrifugation for 30 min at 20,000 g_max. This final pellet (PS), which is referred to as the BBMV fraction, was resuspended (20–30 mg protein/ml) in the same transport loading buffer, dispersed vesiculated by repeated passage through a fine-gauge needle, snap frozen, and stored in liquid nitrogen or used immediately.

In some experiments the P4 fraction was further purified by resuspending and homogenizing the pellet in 50% sucrose (wt/vol), then subjected to density gradient centrifugation (17, 28). The resuspended P4 fraction was layered on top of a cushion of 50% sucrose and overlaid by 40% and then 20% sucrose. This discontinuous sucrose gradient was centrifuged at 190,000 g_max for 90 min at 4°C in an ultracentrifuge. Membrane fractions enriched in basolateral marker enzymes were collected and homogenized in the same buffer used for the initial homogenization.

IT IS WELL ESTABLISHED that the transepithelial transport of the oxalate anion occurs transcellularly, as well as paracellularly, via transport systems located at the apical and basolateral poles of renal and gastrointestinal epithelia (13). One significant aspect of oxalate homeostasis is dependent on the magnitude and direction of oxalate transport across these interfaces with the environment. Although renal excretion of oxalate has long been recognized as a primary means of oxalate loss, the importance of the intestinal handling of oxalate has been generally regarded from the absorptive standpoint, and the possibility of active, transcellular secretion has largely been ignored. That isolated intestinal epithelia can actually secrete oxalate was an incidental observation noted in earlier reports in small (18) and large intestine (14, 15), and we subsequently demonstrated that cAMP stimulates net oxalate secretion across the isolated, short-circuited rabbit proximal (14) and distal colon (15). Because cAMP-stimulated oxalate secretion resembled cAMP-stimulated chloride secretion by the rabbit distal colon in terms of secretagogue stimulation and sensitivity to serosal applied loop diuretics, we hypothesized that cAMP-stimulated oxalate secretion may be mediated by the same, or similar, membrane transport mechanisms that underlie electrogenic chloride secretion by these tissues.
were recovered from the upper interface (20%-40%); membranes enriched in brush-border marker enzymes were harvested from the lower (40%-50%) interface. These fractions were resuspended, syringed, pelleted twice (20,000 gmax, 30 min) using the desired loading buffer, and stored on ice for immediate use.

Marker Enzyme Activity Measurements
Alkaline phosphatase (23) and sucrase (3) were used as apical membrane markers; sodium-potassium-stimulated ATPase activity (4) and potassium-stimulated p-nitrophenylphosphate activity (23) were used as basolateral membrane markers. Other marker enzymes included glucose-6-phosphatase for endoplasmic reticulum (21), thiamine pyrophosphatase as a Golgi membrane marker (30), glucosaminidase as a lysosomal membrane marker (26), and succinate dehydrogenase (25) as a marker for the presence of inner mitochondrial membrane. Pi and glucose were measured with commercially available kits (Sigma Chemical), and protein was determined with Coomassie blue (Bio-Rad), with γ-globulin as a standard.

Uptake of Radioisotopes
Uptake of labeled solutes (36Cl, [14C]oxalate, and [14C]glucose) into membrane vesicles was determined by a rapid filtration method at ambient temperatures (21–24°C, except for results presented in Fig. 7). Uptake was initiated by vortexing a 5–to-10-µl aliquot of vesicle suspension with 45–90 µl of a transport buffer containing radiolabeled solute (compositions of loading and transport buffers are presented for each experiment). Isotope uptake was terminated by adding 1 ml of ice-cold stop buffer (150 mM potassium gluconate, 50 mM mannitol, and 10 mM HEPES titrated to pH 7.4 with Tris base) while vortexing, then pipetting 1 ml of the mixture onto the center of a prewetted filter (0.45-µm pore diameter; HAWP filter, Millipore) that was maintained under a partial vacuum (<200 mmHg). The filter was immediately washed twice with 3 ml of ice-cold stop buffer and dried, and the vesicle contents were solubilized with 4 ml of a scintillation cocktail (Ready Protein, Beckman). Iosotopic activity contained on filters and in transport buffers was quantified by β-scintillation spectrometry at constant quench. Filtration blanks (labeled transport buffer without vesicles) were run routinely along with each experimental series and subtracted from the count rates of individual experimental filters. The radiochemical purity of [14C]oxalate in the transport buffers and vesicle suspensions was assessed by comparing [14C] activity in samples before and after hydrolysis by oxalate decarboxylase (Sigma Chemical) to formate and CO2. Radiochemical purity of oxalate was unaffected after 3 h in the presence of vesicles, indicating that the transported species is oxalate, rather than one of its hydrolysis products.

In most experiments, voltage-sensitive anion uptake was assessed as the difference between uptake measured in the presence and absence of a potassium concentration gradient (plus valinomycin) across the membrane. However, in one series of experiments, diffusion potentials generated by the anion itself were employed to establish voltage dependence of anion transport following the method described by Garty et al. (10). For chloride uptake the P4 fractions were resuspended and homogenized in a buffer containing (in mM) 100 KCl, 100 mannitol, and 20 HEPES-tetramethylammonium (TMA) hydroxide (pH 7.4) for outwardly directed chloride concentration gradients of 100 potassium gluconate, 0.5 TMA chloride, 100 mannitol, and 20 HEPES-TMA hydroxide (pH 7.4) in the absence of a chloride gradient. For oxalate uptake in the presence of an outwardly directed oxalate gradient, the homogenization buffer consisted of (in mM) 50 potassium oxalate, 50 TMA oxalate, and 20 HEPES-TMA hydroxide (pH 7.4) or 100 potassium gluconate, 0.7 TMA oxalate, 100 mannitol, and 20 HEPES-TMA hydroxide (pH 7.4) in the absence of an oxalate gradient. When required, valinomycin incorporation was achieved as described previously before passage through the anion-exchange column. Immediately before use the extravesicular chloride or oxalate was removed by applying a volume (~100 µl) of the loaded vesicles to a small column containing Dowex-1 that had been previously converted to the gluconate form. (Retention of extravesicular oxalate or chloride by the exchange column was complete as measured by the retention of [14C]oxalate or 36Cl applied to the column in the absence of vesicular material.) The BBMVs were eluted from the column with five volumes of 300 mM mannitol-20 mM HEPES-TMA hydroxide (pH 7.4). The last four washes were collected as a single volume and mixed with two volumes of a reaction buffer to give the final extravesicular solute concentrations as described (see Fig. 4). The mixture was stirred at room temperature and immediately spiked (time 0) with TMA-36Cl or TMA2-[14C]oxalate to give final concentrations of 0.5 and 0.7 mM, respectively. Aliquots were removed at given time intervals and then stopped, filtered, and counted as described above.

Transepithelial Flux Measurements
Segments of the distal ileum, stripped of serosal layers, were mounted in Ussing chambers (1.13 cm2) and voltage clamped as described previously (7). Tissues were bathed in 12 ml of buffer containing the following solutes (in mM): 140 sodium, 5.4 potassium, 1.2 magnesium, 1.2 calcium, 123 chloride, 21 HCO3, 2.4 HPO4, 0.6 H2PO4, 10 glucose, and 1.5 × 10−3 oxalate. This buffer was gassed and circulated through water-jacketed reservoirs (37°C) by a gas-lift system (95% O2-5% CO2, pH 7.4). Short-circuit current (Isc, µA·cm−2·h−1) was defined as positive for current flow (positive charge) from the mucosal to the serosal bath, and open-circuit potential (mV) was measured with respect to the mucosal bath. Tissue conductance (mS/cm2) was calculated from the Isc (A/cm2) and open-circuit potential using Ohm's law.

Transepithelial oxalate fluxes across the isolated rabbit ileum were determined as follows. Forty minutes after the tissue was mounted, [14C]oxalate was added to the mucosal or serosal reservoir, and sampling of the opposing reservoir began 20–30 min thereafter. At 10-min intervals the Isc and open-circuit potential were recorded, and 1-ml samples were taken from the appropriate reservoir and replaced with nonlabeled buffer. Samples were dissolved in 5 ml of Ecoscint A (National Diagnostics), and isotopic activity was determined as described above. Unidirectional oxalate fluxes from serosal to mucosal side and mucosal to serosal side were calculated from the difference in activity between successive 10-min samples with correction for the changes in activity due to dilution of the sampling reservoir with nonlabeled buffer.

Chemicals
α-Phenylcinnamate was obtained from Aldrich Chemical. Diphenylamine-2-carboxylate (DPC), DIDS, and 5-nitro-2-(3-phenylpropylamino)benzoate (NPPB) were from Research Biochemicals International. All other chemicals were obtained from Sigma Chemical. Radioisotopes (H36Cl, [14C]oxalate, and d-[14C(U)]glucose) were obtained from New England Nuclear.
Statistics

Each point represents the average of n separate membrane preparations performed in triplicate unless otherwise noted. SE values are based on the number of separate membrane preparations. Comparisons between two means were made using Student’s t-test (unpaired); multiple comparisons were made using a one-way ANOVA followed by a post hoc analysis using the Student-Newman-Keuls method for pairwise comparisons or Bonferroni’s t-test for multiple comparisons with a control. In all cases, differences were considered statistically different if P ≤ 0.05.

RESULTS

cAMP-Stimulated Oxalate Secretion by Rabbit Ileum

Before evaluating possible conductive pathways for oxalate in rabbit ileal membrane vesicles, we first confirmed the suitability of this tissue by establishing the transepithelial handling of oxalate in response to secretagogue. As shown in Fig. 1, addition of dibutyryl cAMP (0.5 mM, serosal) to the isolated, short-circuited rabbit ileum stimulated an increase in $I_{sc}$ and initiated a net secretion of oxalate ($\sim$25 pmol·cm$^{-2}$·h$^{-1}$). It is evident that the net secretion is the result of a twofold increase in flux, rather than a depression in the absorptive component. Hence, the rabbit distal ileum has the requisite machinery for the secretion of oxalate and can serve as a useful source of isolated membranes containing the relevant secretory transport pathways.

Marker Enzyme Profiles of Ileal BBMVs

We characterized the BBMV preparation to estimate the degree of cross-contamination by membranes of basolateral and intracellular origin. Figure 2 depicts the enrichment profiles (ratio of enzyme specific activity in P5 to that in the initial homogenate) for marker enzymes associated with various membranous components present in the P5 fraction routinely used here. Brush-border membrane markers were typically enriched 15- to 18-fold, as judged by alkaline phosphatase and disaccharidase (sucrase) activities in the P5 fractions. A small, but significant, enrichment of basolateral membrane was found in the P5 fraction, as judged by the enrichments of potassium-stimulated $p$-nitrophosphatase (2.7 ± 0.5) and sodium-potassium-stimulated ATPase (1.5 ± 0.2) activities. The endoplasmic reticulum-associated enzyme glucose-6-phosphatase was also slightly enriched (1.5 ± 0.2) in this vesicle fraction. Golgi, lysosomal, and mitochondrial membranes were depurified in the P5 fraction, as evidenced by thiamine pyrophosphatase, glucosaminidase, and succinate dehydrogenase enrichment ratios, respectively. The substantial enrichment of apical membrane markers relative to markers for basolateral membrane indicates that this standard preparation (P5) is reasonably free of contamination.

Voltage-Dependent Components of Chloride and Oxalate Uptake by Ileal BBMVs

Potassium diffusion potentials. The voltage dependence of chloride and oxalate uptake into rabbit ileal BBMVs was evaluated in two ways. First, a potassium diffusion potential was generated by establishing a potassium concentration gradient across the vesicle membrane in the presence of the potassium ionophore valinomycin. Imposition of an inside-positive membrane potential ($[K^+]_i$) < extr-
Anion conductances in BBMVs

Cellular potassium ([K₁₀] + valinomycin) stimulated 36Cl (Fig. 3A) and [14C]oxalate (Fig. 3B) uptake compared with uptake by voltage-clamped (0 mV, [K₁] = [K₀] + valinomycin) vesicles. In contrast, generating a nominally inside-negative membrane potential ([K₁] > [K₀] + valinomycin) significantly reduced the rate of uptake of both anions. Bound chloride represented 1% of the equilibrium chloride content and bound oxalate represented <7% of the equilibrium oxalate content at any voltage (data not shown). It is also evident that there is a measurable potassium-gradient stimulation of anion uptake in the absence of oxalate, which suggests the existence of endogenous potassium conductive pathways in these vesicles or some other potassium-gradient-dependent uptake pathway. It is also noteworthy that measured vesicle activities of [14C]oxalate and 36Cl transiently exceed their respective equilibrium activities at 180 min, indicating a small overshoot phenomenon. Comparison of the uptake points obtained at 8 min with those obtained at 180 min indicates a 1.4-fold accumulation for both anions, and these results were statistically significant.

Anion-generated diffusion potentials. Because the voltage-stimulated uptake of chloride and oxalate shown in Fig. 3 could be partially due to potassium-anion cotransport, we evaluated the dependence of anion uptake on diffusion potentials generated by the anion itself (10). If a conductive pathway for an anion exists in the vesicle preparation, then diffusive efflux of the anion from the vesicle interior through its conductance will establish a diffusion potential (inside positive) that should accelerate the uptake of tracer from the extravesicular medium. Voltage clamping the membrane potential to 0 mV ([K₁] = [K₀] + valinomycin) will depress the conductive component, leaving any trans-stimulated anion-exchange modes intact. As shown in Fig. 4A, the presence of an outwardly directed chloride gradient markedly enhances chloride uptake [compared with nongradient conditions, intracellular chloride ([Cl₁] = extracellular chloride ([Cl₀]),)] by a mechanism that is also depressed when the diffusion potential is short circuited (+ valinomycin, [K₁] = [K₀] + 100 mM). The equilibrium activities of chloride were not significantly different in the three groups of vesicles (with vesicle volumes ~1.0 µl/mg protein). The peak magnitude of the valinomycin-induced differential indicates a vesicular chloride uptake of ~0.4 nmol·mg protein⁻¹·8 min⁻¹ that is conductive under the conditions of these experiments.

Similarly, outwardly directed oxalate gradients (intracellular oxalate > extracellular oxalate) stimulated oxalate uptake (Fig. 4A) compared with nongradient conditions (intracellular oxalate = extracellular oxalate), and this gradient-stimulated pathway was reduced when the diffusion potential generated by oxalate efflux was abolished (+ valinomycin, [K₁] = [K₀] + 100 mM). The maximum magnitude of the valinomycin-induced differential in gradient-driven oxalate uptake indicates a vesicular oxalate uptake of 0.22 nmol·mg protein⁻¹·8 min⁻¹, which is conductive under the conditions of these experiments.

![Fig. 3. Potential dependence of chloride and oxalate uptake by rabbit ileal brush-border membrane vesicles. A: ○, chloride uptake in presence of an inwardly directed potassium gradient with valinomycin (Val). Initial inside-to-outside potassium ratio ([K₁]/[K₀] = 0/100) was generated by mixing vesicles containing (in mM) 100 potassium gluconate, 100 mannitol, and 20 HEPES-TMA hydroxide (pH 7.4); □, chloride uptake by voltage-clamped (0 mV, [K₁] = [K₀] + valinomycin) vesicles. B: ○, chloride uptake in presence of an outwardly directed potassium gradient with Val; □, chloride uptake under the same conditions with Val; ○, chloride uptake in absence of a potassium gradient with Val; □, chloride uptake in presence of an outwardly directed potassium gradient with Val (initial [K₁]/[K₀] = 100/100) achieved by mixing vesicles containing (in mM) 100 potassium gluconate, 100 mannitol, and 20 HEPES-TMA hydroxide (pH 7.4); △, chloride uptake under the same conditions without Val; ○, chloride uptake in presence of an outwardly directed potassium gradient with Val (initial [K₁]/[K₀] = 100/100) achieved by mixing vesicles containing (in mM) 100 potassium gluconate, 100 mannitol, and 20 HEPES-TMA hydroxide (pH 7.4); □, chloride uptake in presence of an outwardly directed potassium gradient with Val (initial [K₁]/[K₀] = 100/100) achieved by mixing vesicles containing (in mM) 100 potassium gluconate, 100 mannitol, and 20 HEPES-TMA hydroxide (pH 7.4); □, chloride uptake in presence of an outwardly directed potassium gradient with Val (initial [K₁]/[K₀] = 100/100) achieved by mixing vesicles containing (in mM) 100 potassium gluconate, 100 mannitol, and 20 HEPES-TMA hydroxide (pH 7.4); □, chloride uptake in presence of an outwardly directed potassium gradient with Val (initial [K₁]/[K₀] = 100/100) achieved by mixing vesicles containing (in mM) 100 potassium gluconate, 100 mannitol, and 20 HEPES-TMA hydroxide (pH 7.4); □, chloride uptake in presence of an outwardly directed potassium gradient with Val (initial [K₁]/[K₀] = 100/100) achieved by mixing vesicles containing (in mM) 100 potassium gluconate, 100 mannitol, and 20 HEPES-TMA hydroxide (pH 7.4). Final oxalate concentration was 1.1 mM. Values are means ± SE of 5 preparations, except △, wherein n = 3.

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Further Purification

To strengthen the conclusion that these voltage-dependent anion transport pathways are principally derived from apical membrane components, we further purified the P5 (P4) fraction by isopycnic and differential centrifugation to resolve an enriched apical membrane fraction and a basolateral membrane fraction. We then compared the transport properties and marker enzyme patterns in the same fractions to test the degree of coenrichment of apical markers and voltage-dependent anion transport. As shown in Fig. 5A, the apical membrane fraction recovered from the 40–50% interface of the discontinuous sucrose gradient was further significantly enriched 1.30-fold (compared with the P5 fraction) in alkaline phosphatase activity while being deenriched (0.51-fold) in sodium-potassium-ATPase activity. In contrast, the enzyme activities associated with the fraction recovered from the upper 20%-40% interface were deenriched (0.16-fold) in alkaline phosphatase but enriched (2.20-fold) in sodium-potassium-ATPase activity, attesting to their basolateral origin. To confirm the distinct origins of the two membrane fractions using a nonenzymatic method, we measured sodium-dependent and sodium-independent glucose uptake in the P5 fraction and in the additionally purified fractions, as shown in Fig. 5B. Sodium-dependent glucose uptake, generally associated with apical membrane, was significantly enriched (1.37-fold) in the apical fraction and deenriched (0.22-fold) in the basolateral membrane fraction compared with the P5 fraction. In contrast, the sodium-independent component of glucose uptake, a characteristic of basolateral membrane glucose transport, was deenriched (0.43-fold) in the apical fraction and enriched (1.30-fold) in the basolateral fraction. Because the enzyme profiles and glucose transport profiles indicate that the apical fraction does indeed exhibit reduced contamination by basolateral membrane material compared with the P5 fraction, the voltage-dependent anion pathways should be reciprocally purified/depurified in the apical/basolateral membrane fractions. As shown in Fig. 5, C and D, potential-dependent chloride and oxalate uptake in apical membrane fractions is significantly enriched (1.78- and 1.48-fold, respectively) compared with the P5 fraction. Furthermore, the potential-sensitive components for chloride and oxalate are significantly reduced in the basolateral fraction compared with the P5 fractions (0.57- and 0.14-fold, respectively) and reduced in the basolateral fraction compared with the apical membrane fractions (0.41- and 0.10-fold, respectively). We conclude from these observations that the voltage-dependent chloride and oxalate transport pathways observed in the P5 fraction are derived, in large part, from apical membranes of the ileum.

Physical Characterization of the Voltage-Dependent Pathway

Kinetics of voltage-dependent chloride and oxalate uptake. Because carrier-mediated transport systems are often characterized as having a relatively high
affinity for their substrates [i.e., affinity constant (K_m) in the low millimolar range (16)], a determination of the concentration dependence of voltage-dependent chloride and oxalate uptake could provide some insight as to the nature (mediated vs. nonmediated) of these anion transport pathways. Figure 6 summarizes the concentration dependence of voltage-dependent chloride (Fig. 6A) and oxalate (Fig. 6B) uptake (5 s) by rabbit ileal BBMVVs, again computed as the difference between uptake in the presence ([K]_o < [K]_o + valinomycin) and absence ([K]_o = [K]_o + valinomycin) of an inwardly directed potassium gradient. Kinetic constants for potential-dependent chloride uptake, derived from a nonlinear curve fit (SigmaPlot 2.0) assuming Michaelis-Menten kinetics, gave a maximal velocity (V_max) of 24.1 ± 1.5 nmol·mg protein⁻¹·5 s⁻¹ and an apparent K_m of 141 ± 17 mM. As shown in Fig. 6B, the voltage-dependent uptake of oxalate, like that of chloride, appeared to exhibit a slight degree of saturability. The apparent overall K_m for voltage-dependent oxalate uptake was estimated to be 126 ± 8 mM, and V_max was 9.6 ± 0.5 nmol·mg protein⁻¹·5 s⁻¹ as determined from the nonlinear curve-fit analysis. We conclude from these kinetic studies that the voltage-dependent components of chloride or oxalate uptake in rabbit ileal BBMVVs exhibit the characteristics of a simple conductance (poorly saturable or low affinity) as opposed to an electrogenic carrier-mediated transport system (saturable or high affinity).

Temperature dependence. We also evaluated the temperature dependence of the voltage-dependent components of chloride and oxalate uptake to test the hypothesis that the voltage-dependent anion uptake pathway(s) characterized previously represents a simple conductive pathway(s) or an electrogenic, carrier-mediated system(s). We measured the temperature dependence of chloride and oxalate uptake into P5 vesicles at 5 s in the presence ([K]_o > [K]_o + valinomycin) and absence ([K]_o = [K]_o + valinomycin) of an inwardly directed potassium gradient and used the difference between these two conditions as a measure of voltage-dependent anion uptake. As shown in Fig. 7, the logarithm of the voltage-dependent uptake velocity for chloride and oxalate was a linear function of the reciprocal of absolute temperature. E_A values (kcal/mol) for the voltage-dependent uptake of chloride and oxalate were derived from the slopes of these plots using the formalism of the Arrhenius relation: log uptake velocity = (−E_A/(2.3R)·(1/T)) + log A, where R is the gas constant (1.98 cal·mol⁻¹·°K⁻¹), T is the absolute temperature (°K), and A is an integration constant. The calculated E_A was 4.7 kcal/mol for voltage-dependent chloride uptake and 6.3 kcal/mol for oxalate, indicating that the transport processes underlying the
voltage-sensitive uptake of these anions are relatively insensitive to temperature (i.e., an $E_A$ of 5 kcal/mol gives a $Q_{10}$ of 1.3 at 20°C). In contrast, $E_A$ for the carrier-mediated chloride/bicarbonate exchange system present in these vesicles (17) was 16.5 kcal/mol (Fig. 7A) and that for carrier-mediated oxalate/bicarbonate exchange (19) was 19.8 kcal/mol (Fig. 7B). Additionally, the $E_A$ measured for sodium-dependent d-glucose uptake was 24.1 kcal/mol (data not shown) when measured as the difference between 5-s uptakes in the presence and absence of extravesicular sodium. On the basis of these $E_A$ measurements, we conclude that the voltage-dependent pathway(s) for chloride and oxalate described here are similar to aqueous pores or channels, rather than a carrier-mediated system(s).

Anion Transport Inhibitors

The final approach to characterize the nature of the voltage-sensitive anion transport was to establish inhibitor sensitivity profiles for anion uptake into BBMVs using the P5 fraction. Figure 8 depicts the effects of seven inhibitors on potential-dependent chloride and oxalate uptake that have previously been shown to block chloride conductances in other preparations (2, 9). At 250 µM, neither tamoxifen, the stilbene SITS, nor α-phenylcinnamate significantly reduced chloride (Fig. 8A) or oxalate (Fig. 8B) uptake compared with vehicle (0.5% DMSO)-treated controls. Interestingly, voltage-sensitive uptake of these anions are relatively insensitive to temperature (i.e., an $E_A$ of 5 kcal/mol gives a $Q_{10}$ of ~1.3 at 20°C). In contrast, $E_A$ for the carrier-mediated chloride/bicarbonate exchange system present in these vesicles (17) was 16.5 kcal/mol (Fig. 7A) and that for carrier-mediated oxalate/bicarbonate exchange (19) was 19.8 kcal/mol (Fig. 7B). Additionally, the $E_A$ measured for sodium-dependent d-glucose uptake was 24.1 kcal/mol (data not shown) when measured as the difference between 5-s uptakes in the presence and absence of extravesicular sodium. On the basis of these $E_A$ measurements, we conclude that the voltage-dependent pathway(s) for chloride and oxalate described here are similar to aqueous pores or channels, rather than a carrier-mediated system(s).
dependent uptake of chloride and oxalate were significantly stimulated by tamoxifen. On the other hand, chloride and oxalate uptake were significantly reduced by the stilbene DIDS, the nonsteroidal anti-inflammatory flufenamate, and the more conventional inhibitors of chloride conductance DPC and NPPB (assuming that the inhibitors are not affecting the driving force). The relative changes in chloride and oxalate uptake (right ordinates in Fig. 8) produced by the inhibitors suggest that the inhibition of potential-dependent oxalate uptake is somewhat greater than that of chloride.

**DISCUSSION**

The present studies demonstrate that the isolated, short-circuited rabbit distal ileum is capable of secreting the divalent organic anion oxalate in response to a secretory stimulus by a mechanism that primarily involves an upregulation in the serosal-to-mucosal unidirectional oxalate flux across the tissue. In this manner, ileal oxalate secretion is similar to that observed in the rabbit distal colon (15). In the latter study, the cAMP-stimulated oxalate secretion was inhibited by serosally applied bumetanide and partly inhibited by mucosal addition of the chloride channel blocker NPPB. Because the characteristics of oxalate secretion by these tissues resembled that of cAMP-stimulated chloride secretion, we proposed (15) that the oxalate anion may utilize pathways that are the same as or similar to those used by chloride (12).

A conductive pathway for oxalate uptake was not previously detected in brush-border membranes prepared from the rabbit distal ileum (18); however, conductive pathways for chloride in small intestinal brush-border membranes have been identified in preparations derived from rat (20) and rabbit (17) ileum, rabbit duodenum (1), and pig jejunum (5). In the last study (5), it was concluded that the nature of the buffers employed is an important consideration in detecting conductive pathways for chloride, since vesicles prepared in imidazolium acetate or HEPES-Tris buffers lacked a chloride conductance, whereas those made in HEPES-TMA buffers exhibited a significant chloride conductance. This striking buffer dependence may partly explain the reported absence of a voltage-dependent component of oxalate uptake by rabbit ileal brush-border vesicles (18), since the solutions employed in that study were buffered with 96 mM HEPES plus 50 mM Tris.

A voltage dependence is not sufficient to distinguish an electrogenic, carrier-mediated transport process from a channel- or pore-type mechanism. Three independent lines of evidence obtained in the present study suggest that the voltage-dependent uptake of chloride and oxalate occurs by a channel- or pore-type mechanism. First, under conditions where \([K]_i = [K]_o\) (Fig. 4), inclusion of valinomycin depressed the uptake of chloride or oxalate in the presence of an outwardly directed gradient for the respective anion. Although trans-stimulation by the respective anion might also be expected for an anion-exchange process under these experimental conditions, depression of part of the uptake process by valinomycin (i.e., short-circuiting the vesicle) implies that the membrane potential is generated by gradient-driven, diffusive efflux of the anion from the vesicle, in other words, a conductance.

A second feature of the voltage-sensitive uptake of chloride and oxalate that is more characteristic of a conductance than a carrier-mediated transporter is the observation that the voltage-dependent uptake of both anions was only weakly saturated by increasing concentrations of the respective anion. The \(K_m\) values estimated from the concentration dependence for voltage-sensitive anion uptake were \(141 \pm 17\) and \(126 \pm 8\) mM for chloride and oxalate, respectively. A low affinity (\(K_m = 116\) mM) has also been ascribed to the chloride conductance present in brush-border membranes prepared from the pig jejunum under conditions supporting a chloride conductance (5). In contrast, reported \(K_m\) values for chloride or oxalate exchangers (17, 19) are one to two orders of magnitude smaller than the voltage-sensitive components measured here and in pig jejunal brush-border vesicles (5). Although the vesicle...
estimates for \( K_m \) of the voltage-sensitive pathways presented here are on the low end of ionic conductances measured using electrophysiological methods (11, 16), interpreting these results in terms of a pore or channel model is reasonable, particularly in light of the temperature dependence of these transport pathways (see below).

The third characteristic that suggests the voltage-dependent uptake of oxalate and chloride is channel mediated is the low apparent \( E_A \) measured for each transport process (6.3 and 4.7 kcal/mol for oxalate and chloride, respectively). For chloride, these results alone suggest that the voltage-dependent component represents diffusion through an aqueous pore or channel, since the \( E_A \) is similar to free solution diffusion of chloride (16) and the process measured here has a temperature dependence similar to the apical, 40-pS chloride conductance in T84 cells [\( E_A \) of 3.6-4.7 kcal/mol at -80 and +80 mV clamp potential (11)]. We expect that the same is true for the oxalate anion, given the low \( E_A \) measured for voltage-dependent oxalate uptake. Generally, conductance of an ion through a channel exhibits a temperature dependence akin to that for ion diffusion in aqueous solutions (16), as opposed to carrier-mediated or enzymatic processes, which often exhibit \( E_A \) values that are severalfold greater because of the nature of the interactions between the carrier and its substrate. This distinction was clearly demonstrated in the present study, where the \( E_A \) values for chloride/bicarbonate and oxalate/bicarbonate exchange and sodium-dependent D-glucose uptake by brush-border vesicles were 16.5, 19.8, and 24.1 kcal/mol, respectively. On the basis of kinetic and thermodynamic properties, we conclude that the voltage-sensitive uptake of oxalate and chloride under the present experimental conditions can be best described as channel-mediated conductances, rather than electrogenic carrier-mediated systems.

Although the conductances for oxalate and chloride can be defined solely by their physical properties, studies presented here using a series of putative chloride channel blockers further support the notion that anion conductances are present in rabbit ileal BBMVs. Voltage-dependent uptake of oxalate and chloride were reduced by the stilbene DIDS (but not SITS), by the nonsteroidal anti-inflammatory flufenamate, and by the phenyl carboxylates DPC and NPPB. Phenylcinna- mide, an anion conductance blocker in pig jejunal brush-border vesicles (6), reduced anion uptake, but not significantly at the concentration employed (250 \( \mu \)M). Interestingly, tamoxifen, an antiestrogenic compound that reportedly blocks volume-sensitive chloride channels (24), actually increased anion conductance in this series. A similar effect has been recently noted in patch-clamp studies of this channel in endothelial cells (22). Although chloride channel blockers did reduce anion conductances in our preparation, no significant profile emerged; all effective agents reduced chloride conductance by -50% and oxalate conductance 35-40%. One interpretation of these results is that chloride and oxalate share a common conductance pathway (same broad profile of inhibition), and, in the presence of a blocking agent, oxalate conductance is reduced to a slightly greater degree than chloride conductance. We have not further explored the possibility of a common pathway using competition experiments because no unique outcome could be expected that would distinguish between one or two (or more) conductances, since the anion fluxes are electrically coupled in any experimental scenario. Given the broad selectivity of chloride channels, as considered below, the hypothesis that the oxalate conductance observed here is mediated by a chloride conductive pathway remains a distinct possibility.

An important question arising from the conclusion that the voltage-dependent uptake of oxalate and chloride is channel mediated concerns the nature of the chloride conductance characterized here. Chloride channels in secretory epithelia have been segregated into three general classes on the basis of their activation patterns, conductance, and, to a lesser degree, their sensitivity to channel blockers (8, 9, 24, 29). Agonist-activated chloride channels are stimulated by cAMP (\( G_{Cl}^{cAMP} \)) or Ca (\( G_{Cl}^{Ca} \)); volume-activated chloride channels (\( G_{Cl}^{OR} \)) respond to deformation of the membrane [also referred to as the volume-sensitive outward rectifier (VSOR)] (24, 29), and the outwardly rectifying chloride channel (\( G_{Cl}^{OR} \)) is activated experimentally by patch excision (9). Anion selectivity of these channels in general tends to be low, and this is particularly apparent in \( G_{Cl}^{OR} \) and \( G_{Cl}^{OR} \), for which detailed patterns of halide as well as organic anion selectivity have been established. For example, the VSOR channel(s) mediates the conductive efflux of a large number of structurally disparate organic solutes, such as taurine, inositol, and even gluconate (24, 29), whereas the 40-pS, outward rectifier present in T84 cell apical membranes (\( G_{Cl}^{OR} \)) exhibits a permeability to formate, bicarbonate, and lactate anions of 0.76, 0.44, and 0.30, respectively, relative to chloride (11). On the basis of these observations, the possibility that oxalate could be conducted through \( G_{Cl}^{OR} \) or \( G_{Cl}^{OR} \) appears strong, and it would not be particularly surprising if the agonist-activated chloride channels also have a measurable oxalate conductance.

Our initial hypothesis was that the cAMP-stimulated, transepithelial secretion of oxalate was mediated apically by an agonist-activated conductance pathway (\( G_{Cl}^{cAMP} \)) similar to that for chloride. Yet each of the chloride channel types considered above might be considered a candidate mechanism for the chloride (oxalate) conductance detected in the current vesicle studies. If inhibitor sensitivities and ATP dependence are considered and if it is assumed that channel integrity is fundamentally similar membrane patches and vesicles, then \( G_{Cl}^{OR} \) emerges as the most likely pathway mediating vesicle anion conductances observed here. The inhibition by DIDS might exclude \( G_{Cl}^{OR} \), since this conductance is not particularly sensitive to this stilbene (9), and the location of an inhibitory effect of tamoxifen excludes \( G_{Cl}^{OR} \). Since low doses of this drug block anion efflux and conductance (24), a nucleotide dependence also is a prominent requirement for sustaining \( G_{Cl}^{OR} \) (24, 29) and probably \( G_{Cl}^{cAMP} \) (9); hence, absence of
ATP in the media used here might further preclude these as possible mediators of anion conductance in the present system. A comparable analysis of $G_{\text{Cl}}^{\text{FR}}$ as a contributor to vesicle anion conductance is hampered by a relative lack of information regarding inhibitor sensitivities, but the fact that these channels exhibit significant rundown after excision (9) again suggests a requirement for some cytosolic component that is absent in the vesicle system. In contrast, $G_{\text{Cl}}^{\text{FR}}$ is independent of cytosolic factors (including ATP), is inhibited by DPC and DIDS, and is actually activated by patch excision (9), a configuration that more closely resembles the intra- and extravascular milieu employed in studies using brush-border membrane preparations. The extent to which $G_{\text{Cl}}^{\text{FR}}$ participates in epithelial anion secretion is arguable (9), yet there is some evidence that this chloride channel may also be regulated by the cystic fibrosis transmembrane conductance regulator gene product and contributes to whole cell currents stimulated by cAMP (27). Hence, if the anion conductances for oxalate and chloride observed in rabbit ileal vesicles are mediated by $G_{\text{Cl}}^{\text{FR}}$, then the possibility remains that chloride and oxalate fluxes through this channel contribute to the cAMP-stimulated secretion of these anions across the rabbit ileum. Although it is unlikely that specific channels have evolved to handle anions such as oxalate, exploitation of the broad selectivity of chloride conductive pathways may represent an economical strategy in the homeostasis of this important anion.

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