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The Nature of the Neutral Na^+-Cl^- Coupled Entry at the Apical Membrane of Rabbit Gallbladder Epithelium: III. Analysis of Transports on Membrane Vesicles

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Summary. In rabbit gallbladder epithelium, a Na^+/H^+, Cl^-/HCO_3^- double exchange and a Na^+-Cl^- symport are both present, but experiments on intact tissue cannot resolve whether the two transport systems operate simultaneously. Thus, isolated apical plasma membrane vesicles were prepared. After preloading with Na^+, injection into a sodium-free medium caused a stable intravesicular acidification (monitored with the acridine orange fluorescence quenching method) that was reversed by Na^+ addition to the external solution. Although to a lesser extent, acidification took place also in experiments with an electric potential difference (PD) equal to 0. If a preset pH difference (ΔpH) was imposed ($[H^+]_{in} > [H^+]_{out}$, PD = 0), the addition of Na-gluconate to the external solution caused ΔpH dissipation at a rate that followed saturation kinetics. Amiloride (10^{-4} M) reduced the ΔpH dissipation rate. Taken together, these data indicate the presence of Na^+ and H^+ conductances in addition to an amiloride-sensitive, electroneutral Na^+/H^+ exchange.

An inwardly directed $[Cl^-]$ gradient (PD = 0) did not induce intravesicular acidification. Therefore, in this preparation, there was no evidence for the presence of a Cl^-/OH^- exchange.

When both $[Na^+]$ and $[Cl^-]$ gradients (outwardly directed, PD = 0) were present, fluorescence quenching reached a maximum 20–30 sec after vesicle injection and then quickly decreased. The decrease was not observed in the presence of a $[Na^+]$ gradient alone or the same $[Na^+]$ gradient with Cl^- at equal concentrations at both sides. Similarly, the decrease was abolished in the presence of both Na^+ and Cl^- concentration gradients and hydrochlorothiazide (5×10^{-4} M). The decrease was not influenced by an inhibitor of Cl^-/OH^- exchange (10^{-4} M furosemide) or of Na^+-K^+-$2Cl^-$ symport (10^{-5} M bumetanide).

We conclude that a Na^+/H^+ exchange and a Na^+-Cl^- symport are present and act simultaneously. This suggests that in intact tissue the Na^+-Cl^- symport is also likely to work in parallel with the Na^+/H^+ exchange and does not represent an induced homeostatic reaction of the epithelium when Na^+/H^+ exchange is inhibited.

Key Words gallbladder · apical membrane vesicles · Na^+/H^+ exchange · Cl^-/OH^- exchange · Na^+-Cl^- symport · furosemide · bumetanide · hydrochlorothiazide

Introduction

A coupled, electrically neutral, sodium and chloride absorption occurs across the apical membrane of gallbladder epithelium. The coupling has been explained by a simple Na^+-Cl^- symport on a single carrier or by a Na^+/H^+, Cl^-/HCO_3^- dual exchange (Heintze, Petersen & Wood, 1981; Reuss, 1984; Spring, 1984; Cremaschi et al., 1987*a,b*). Available data suggest differences among animal species.

In rabbit gallbladder, electrophysiologic experiments as well as radioisotopic measurements in intact epithelium have indicated the presence of a dual exchange and a Na^+-Cl^- symport which are not affected by K^+ or bumetanide (Cremaschi, Meyer & Rossetti, 1983; Cremaschi et al., 1987*a,b*). Nevertheless, evidence has been obtained in recent years that in many epithelial and nonepithelial cells the Na^+-Cl^- symport is, in fact, a Na^+-K^+-$2Cl^-$ symport (Hannafin et al., 1983; Koenig, Ricapito & Kinne, 1983; Oberleithner et al., 1983; Frizzell & Field, 1984; Greger et al., 1984; Brown & Murer, 1985; Epstein & Silva, 1985; Turner, George & Baum, 1986; O'Grady, Palfrey & Field, 1987). A true Na^+-Cl^- symport seems to be present only in few cell types besides rabbit gallbladder epithelium (Costanzo & Windhager, 1978; Frizzell & Field, 1984; Stokes, 1984; Velazquez, Good & Wright, 1984). Indeed, it has also been suggested that Na^+-K^+-$2Cl^-$ and Na^+-Cl^- symports could represent different functional moments of the same transport system (Eveloff & Calamia, 1986; Hoffman, 1986). The two symports, in addition to playing important roles in transepithelial transport, appear to be related to cell volume regulation. The latter has been observed in Erlich ascites tumor cells (Hoffman, Sjoholm & Simonsen, 1983; Geck & Pfeiffer, 1985), the thick ascending limb of Henle's loop (Eveloff & Calamia, 1986), frog skin (Ussing, 1982, 1985), avian red cells (Ueberschar & Bakker-Grunwald, 1983) and human fibroblasts (Owen & Prastein, 1985) after hyperosmotic cell shrinkage. Therefore, it follows that the Na^+-Cl^- symport revealed in rabbit gallbladder could operate simultaneously with dual exchange or be transiently activated as a homeostatic reaction of the cell owing to the experimental conditions that

abolished Na^+/H^+, Cl^-/HCO_3^- exchange. On the basis of experiments performed on intact tissue, both hypotheses remain valid (Cremaschi et al., 1987*a*). Since we recently developed a method to isolate apical plasma membranes in rabbit gallbladder epithelium (Bottà et al., 1987), we were able to set up experiments with vesicles to characterize ion-translocating mechanisms present on the apical membrane free of cytoplasmic control.

Materials and Methods

Membrane Preparation

Brush border membrane vesicles (BBMV) were prepared from New Zealand rabbit gallbladders collected directly in a slaughterhouse (Azienda Agricola Bernasconi, Valmorea, Como, Italy). Gallbladders, excised immediately after the animal was killed by electroshock, were washed free of bile with a Krebs-Henseleit solution; the mucosa was scraped off and thereafter kept in a Tris-mannitol solution at 0–4°C. Scraped mucosae, brought to our laboratories within 60–80 min, were successively subjected to $CaCl_2$ or, if specified, to $MgCl_2$ treatment followed by isopycnic centrifugation in a Percoll medium, as already described (Bottà et al., 1987). To obtain and preload vesicles, the pellet obtained after eliminating the residual Percoll was resuspended in an appropriate buffered saline (i.e., the loading solution) for 8–10 hr at 4°C and then centrifuged at 120,000 × *g* for 45 min. The final pellet was resuspended, using a syringe and fine needle, in the same saline and left for 2 hr at room temperature before experimentation. Protein concentration was measured by a Bio-Rad assay kit and adjusted to a final value of 6.5 mg/ml.

Measurement of ΔpH

Changes in transmembrane pH gradients were analyzed using the fluorescence quenching of acridine orange (AO) (Warnock, Reenstra & Yee, 1982). Nonconventional use of a microfluorimeter (Leitz MPVII-Ploemopak) allowed us to inject very small samples (1 μl) of BBMV into 71 μl of the test solution, which already contained AO at a final concentration of 6 μM (protein final concentration, 90.3 mg/ml). The container was a small cylinder (diameter, 5 mm; height, 2 mm) drilled in a thin steel plate. Solutions were continuously stirred by a microstirrer. The laboratory containing all the measurement apparatuses was thermostated to keep all solutions at 20 ± 1°C. Selection of excitation (450 nm < λ < 490 nm) and emission light (λ > 515 nm) was accomplished using a Leitz filter set (I2) comprising an excitation filter (BP 450–490), a dichroic plate (RKP510) and an emission filter (LP 515). Measuring and illuminating fields were circular (150 and 380 μm diameters, respectively). Fluorescence of the test solution containing AO was arbitrarily adjusted to 100 units before vesicle addition. Fluorescence variations were calculated as a percentage with reference to the level attained after BBMV injection into the test solution containing AO in the absence of a ΔpH. In this way, results do not depend on the nonspecific quenching, which is ΔpH independent and due to vesicle addition (*see* Results and Warnock et al., 1982). Curves shown in the figures are from single experiments, and each trace is the most representative of the average behavior of many separate preparations (three determinations for each preparation). Superimposition of many traces obtained in different conditions has been done in several cases for an easier comparison of the results of different experiments. The number of preparations for each experimental condition is reported, together with relevant statistical data (*see also* Table 1).

To evaluate initial Na^+-dependent rates of dissipation of an imposed pH gradient, we considered the interval between 0.5 and 3 sec after injection of Na-gluconate or N-methylglucamine-gluconate.

Bathing Solutions

Solutions had the following compositions (in mM): (i) Krebs-Henseleit: 142.9 Na^+, 5.9 K^+, 2.5 Ca^{2+}, 1.2 Mg^{2+}, 127.7 Cl^-, 24.9 HCO_3^-, 1.2 $H_2PO_4^-$, 1.2 SO_4^{2-}, pH 7.8 (this pH value has been found to give the best results in order to wash gallbladders free of bile). (ii) Tris-mannitol: 10 Tris, 300 mannitol, pH 7.1. Different solutions used as intra- and extravesicular media contained NaCl, Na-gluconate, K-gluconate, N-methylglucamine-Cl (*see* figure legends), all brought to a final osmolality of 308 mosmol/kg by the addition of N-methylglucamine-gluconate. The pH was 7.1 if not otherwise specified. The buffer was 1 mM HEPES/Tris. When needed, 5 μM valinomycin in the external medium together with intra- and extravesicular 50 mM [K^+] allowed clamping of the voltage to zero. In some of the experiments, valinomycin was added to the suspension that contained vesicles 1 hr before its utilization. Results were not influenced by such a pretreatment, which suggests a fast partition of valinomycin in the membrane.

Statistics

Data are reported as mean ± SE followed by the number (*n*) of separate BBMV preparations. Each preparation was obtained from over 100 gallbladders; at least three different determinations with the same preparation were made for each experimental condition. Statistical significance was evaluated with the paired or the unpaired *t* test.

Materials

AO, valinomycin, nigericin, monensin, furosemide and hydrochlorothiazide (HCTZ) were obtained from Sigma Chemicals (St. Louis, MO); bumetanide was a kind gift from Leo Pharmaceutical Products (DK-2750 Ballerups, Denmark) and amiloride from Merck Sharp & Dohme (Rahway, NJ). All other chemicals were reagent grade. N-methylglucamine-gluconate was obtained by titration of the gluconic acid.

Results

ΔpH Dependence of AO Fluorescence Quenching

Dye response calibration was accomplished by applying various ΔpH across vesicle membranes. Internal pH was fixed at a value of 5. The drop in

Table 1. Maximal initial fluorescence quenching (subtracted for nonspecific quenching) and ΔpH-dependent fluorescence recovery ($\Delta F\%$ obtained 30 sec or 3 min after vesicle injection) in different conditions[a]

Experimental conditions	$\Delta F\%$ maximal initial quenching	$\Delta F\%$ recovery	
		30 sec	3 min
$\uparrow Na^+$	28.9 ± 4.3 (4)		27.9 ± 4.8 (4)
$\uparrow Na^+$ (PD = 0)	10.5 ± 2.8 (5)	10.4 ± 1.5 (5)	9.6 ± 4.0 (5)
$\downarrow Cl^-$	40.1 ± 3.1 (4)		39.5 ± 2.7 (4)
$\downarrow Cl^-$ (PD = 0; pH = 7.1)	0.1 ± 0.1 (4)		0.1 ± 0.1 (4)
$\downarrow Cl^-$ (PD = 0; pH = 8.0)	0.1 ± 0.2 (4)		0.2 ± 0.1 (4)
$\downarrow Cl^- + \downarrow K^+$; nigericin 20 μM (PD = 0; pH = 8.0)	46.9 ± 3.7 (4)		46.5 ± 3.5 (4)
$\uparrow Na^+ + \uparrow Cl^-$ (PD = 0)	7.2 ± 1.4 (11)	7.5 ± 0.9 (11)	1.5 ± 0.5 (11)
$\uparrow Na^+ + \uparrow Cl^-$; furosemide 10^{-4} M (PD = 0)	7.4 ± 1.8 (4)	7.7 ± 1.9 (4)	1.6 ± 1.0 (11)
$\uparrow Na^+ + \uparrow Cl^-$; bumetanide 10^{-5} M (PD = 0)	7.1 ± 1.2 (4)		1.9 ± 0.8 (4)
$\uparrow Na^+$ (PD = 0; $Cl^-_{in,out}$ = 100 mM)	11.1 ± 1.5 (5)		9.3 ± 2.0 (5)
$\uparrow Na^+ + \uparrow Cl^-$; HCTZ 5×10^{-4} M (PD = 0)	11.2 ± 1.7 (4)		10.3 ± 3.0 (4)

[a] Values of maximal initial fluorescence quenching refer only to the experiments whose recovery value is that taken at 3 min. ↑, ↓ stand respectively for an outwardly or an inwardly directed concentration gradient (100/0 mM).

fluorescence observed on addition of vesicles was related to the preset ΔpH in a range between 1 and 2.5 units. After subtraction of the small nonspecific quenching detected by equilibrating internal and external pH, we found a linear correlation between the percentage of fluorescence change (y) and ΔpH (x): $y = 33.9 \pm 4.9x - 19.9 \pm 3.6$ ($r = 0.91 \pm 0.04$; $n = 7$). The intercept on the x axis indicated that pH variations should not be detectable unless the difference between extra- and intravesicular pH was at least about 0.6 units.

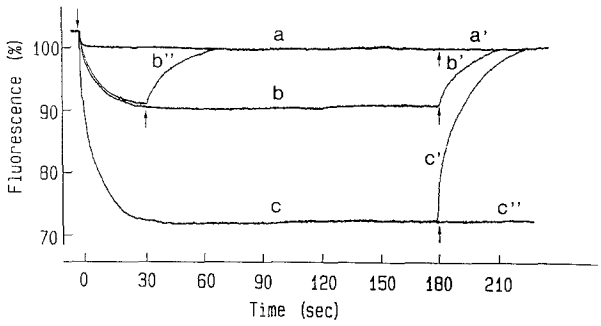

Fig. 1. Changes in intravesicular pH in response to an outwardly directed [Na^+] gradient. ↓ indicates injection of brush border membrane vesicles (BBMV). Composition of initial intra- (in) and extravesicular (out) media refers to each trace. Trace *a*: in = out = 100 mM Na^+, pH 7.1. Trace *b*: in = 100 mM Na^+, 50 mM K^+, pH 7.1; out = 0 mM Na^+, 50 mM K^+, 5 μM valinomycin, pH 7.1. Trace *c*: in = 100 mM Na^+, pH 7.1; out = 0 mM Na^+, pH 7.1. Solutions were maintained isotonic and at the desired initial pH as specified in Materials and Methods. All the external solutions contained 6 μM acridine orange. ↑ indicates injection of Na-gluconate (final concentration = 28 mM, traces *a'*, *b'*, *b''*, *c'*) or N-methylglucamine-gluconate (final concentration = 28 mM, trace *c''*). Each trace has been chosen to be representative of the average time course of each experiment

Transvesicular ΔpH Dependent on a Na^+ Concentration Gradient

To reveal ionic movements that might be coupled to OH^- or H^+, we tested the effects of preimposed ionic concentration gradients on intravesicular acidification.

When vesicles preloaded with 100 mM Na^+ were injected into a medium with the same composition and pH, there was a small fluorescence quenching (Fig. 1, trace *a*) that was not modified following the addition of a protonophore (5 μM monensin or 5 μM

monensin and 28 mM Na^+), of 28 mM Na-gluconate or 28 mM N-methylglucamine-gluconate to the external medium (Fig. 1, trace *a'*). Such quenching corresponded to the small nonspecific ΔpH-independent fluorescence quenching described in the previous section of Results. However, when vesicles preloaded with 100 mM Na^+ were placed in an external medium with the same pH but without Na^+, a marked fluorescence quenching and thus a marked intravesicular acidification took place upon vesicle injection (Fig. 1, trace *c*). Such quenching, except for the nonspecific component, was reversed by Na^+ addition to the external medium. The degree of reversibility depended on the final concentration of the added Na^+ and was maximum at 28 mM Na^+ (Fig. 1, trace *c'*: fluorescence difference (ΔF) = 27.9 ± 4.8%; $n = 4$; $P < 0.01$). Increasing the final Na^+ concentration (up to 56 mM) in the external medium did not cause any more significant fluorescence variation. Addition of N-methylglucamine-gluconate (up to 28 mM) had no significant effect on fluorescence (Fig. 1, trace *c''*).

Maintenance of a null transmembrane PD ($[K^+]_i = [K^+)_o$ = 50 mM + 5 μM valinomycin) reduced but did not prevent fluorescence quenching (Fig. 1, trace *b*). In fact, Na^+-dependent fluorescence recovery observed 3 min after vesicle injection, although smaller than that obtained when K^+ was absent, was significantly different from zero (Fig. 1, trace *b'*: ΔF = 9.6 ± 4.0%; $n = 5$; $P < 0.01$).

The fluorescence quenching observed after vesicle injection attained its maximum at about 30 sec and thereafter decreased very slowly during the following minutes (less than 10% in 3 min) so that Na^+ addition 30 sec after vesicle injection caused a fluorescence recovery (Fig. 1, trace *b''*) that was not statistically different from that obtained at 3 min (*see* Table 1).

Effects of Na^+ and Amiloride on the Dissipation Rate of an Imposed Transvesicular ΔpH

To have an indication of Na^+ fluxes not electrically coupled to H^+, we set up experiments in which a preset ΔpH was present (1.8 pH units) between the inside and the outside of the vesicles. Na^+ was absent and the electric potential difference (PD) was abolished. Vesicle injection caused a rapid initial fluorescence quenching that dissipated only at a very slow rate (Fig. 2*A*). Addition of Na^+ at different concentrations to the external medium 10 sec after vesicle injection increased the rate of dissipation in a concentration-dependent way (Fig. 2*A*, traces *a* and *b*). In contrast, addition of N-methylglucamine-gluconate to the same final concentration values did not modify the rate of spontaneous dissipation (Fig. 2*A*, trace *c*: ΔF spontaneous dissipation = 0.1 ± 0.05% sec^{-1}; $n = 5$).

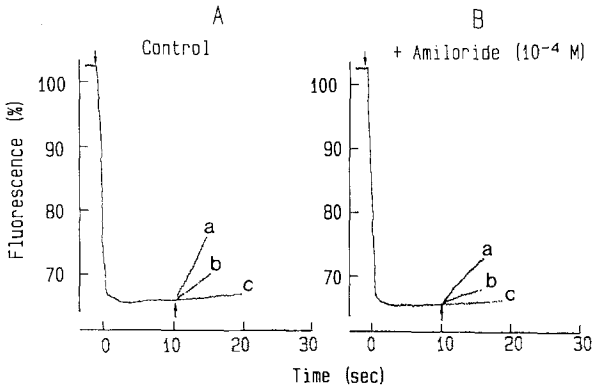

Fig. 2. Effects of sodium on the dissipation of a preset ΔpH in the absence (*A*) or presence (*B*) of amiloride (10^{-4} M) in the external medium. ↓ indicates injection of BBMV. In, out = 0 mM Na^+, 50 mM K^+; in = pH 5.3; out = pH 7.1; out = 5 μM valinomycin. Solutions were maintained isotonic and at the desired initial pH as specified in Materials and Methods. All the external solutions contained 6 μM acridine orange. ↑ indicates injection of Na-gluconate (*a* = 28 mM, *b* = 4.7 mM, final concentrations) or N-methylglucamine-gluconate (*c* = 28 mM, final concentration). Each trace has been chosen to be representative of the average time course of each experiment

Although the relationship between $\Delta F\%$ and intravesicular $[H^+]$ changes is logarithmic (*see* first section of the Results), in the range of the small $\Delta F\%$ variations such as those observed in our experiments it can be reasonably approximated to a linear relationship. Therefore, the rate of dissipation of fluorescence quenching can be assumed as proportional to the H^+ efflux. This conclusion is supported by data from other authors (Sabolic & Burckhardt, 1983*b*; Cassano, Stieger & Murer, 1984), who found a Na^+/H^+ saturation kinetics on the basis of the relationship between $\Delta F\%$ sec^{-1} and Na^+ concentrations with the AO method. The linear dependence of ΔF/sec (with a preset ΔpH and $[K^+]$ at equal concentrations inside and outside) from H^+ efflux was also confirmed by the experiments of Ives, Yee and Warnock (1983), which were based on the use of different concentrations of nigericin added to the extravesicular medium.

The Na^+-dependent dissipation rate exhibited saturation kinetics (K_m = 6.3 ± 0.9 mM, V_{max} = 2.0 ± 0.1 $\Delta F\%$ sec^{-1}; $n = 5$; *see* Fig. 3). Addition of amiloride (10^{-4} M) to the external solution did not alter initial quenching nor its spontaneous dissipa-

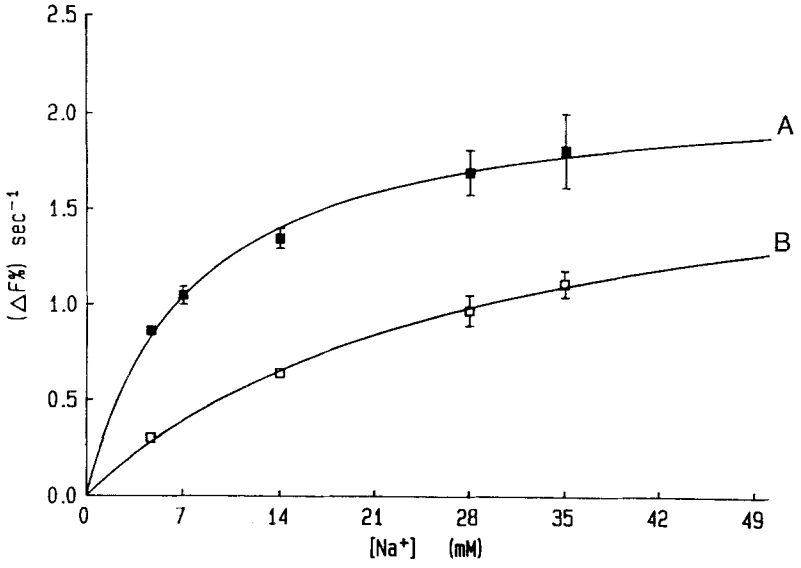

Fig. 3. Kinetics of Na^+/H^+ exchange in BBMV in the absence (*A*) or in the presence (*B*) of 10^{-4} M amiloride. The Na^+-dependent dissipation rates of the fluorescence quenching due to an imposed ΔpH are reported. Each point represents the mean ± SE of the results obtained with 5 (*A*) or 3 (*B*) separate vesicle preparations (three determinations for each preparation)

tion with respect to control (Fig. 2*B*). However, the rate of ΔpH dissipation caused by Na^+ addition, although still concentration dependent, was significantly smaller in amiloride than in control conditions (Fig. 2*B*, trace *a*, $P < 0.01$; trace *b*, $P < 0.02$). The degree of inhibition was inversely proportional to the final $[Na^+]$ (50% inhibition at $[Na^+] = 4.7$ mM; 39% inhibition at $[Na^+] = 28$ mM, *see also* Table 3). Data obtained from experiments with amiloride (10^{-4} M) at various $[Na^+]$ yielded kinetic characteristics (*see* Fig. 3) which differed significantly from those obtained in the absence of the inhibitor only in the K_m value ($V_{max} = 2.0 \pm 0.2$ $\Delta F\%$ sec^{-1}; $K_m = 28.8 \pm 4.1$ mM; $n = 3$).

Transvesicular ΔpH Due to an Inwardly Directed Cl^- Concentration Gradient

An inwardly directed $[Cl^-]$ gradient ($Cl_i = 0$ mM, $Cl_o = 100$ mM) caused marked intravesicular acidification (Fig. 4, trace *c*). Na^+ (28 mM) + monensin (5 μM), added 3 min after vesicle injection, restored initial fluorescence except for nonspecific quenching (Fig. 4, trace *c'*: $\Delta F = 39.5 \pm 2.7\%$; $n = 4$). However, if the electric potential difference was abolished, vesicle injection was not followed by any acidification, nor did subsequent Na^+ addition cause any change in fluorescence (Fig. 4, traces *b* and *b'*: $\Delta F = 0.1 \pm 0.1\%$; $n = 4$; *see also* Table 1). Similar results (Fig. 5, traces *a* and *a'*: $\Delta F = 0.2 \pm 0.1\%$; $n = 4$) were obtained when internal and external pH were higher (pH = 8). To verify vesicle integrity

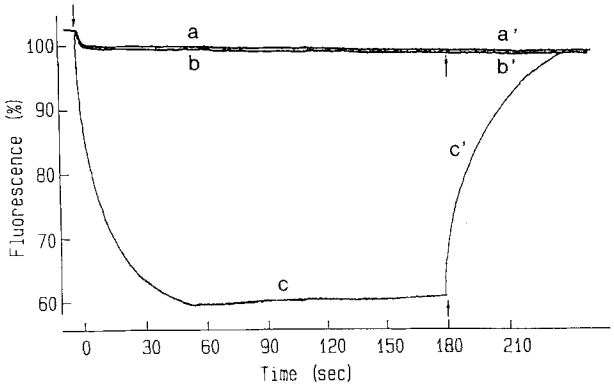

Fig. 4. Intravesicular pH variations dependent on an inwardly directed $[Cl^-]$ gradient. ↓ indicates injection of BBMV. Trace *a*: in, out = 0 mM Na^+, 0 mM Cl^-, 50 mM K^+, pH 7.1. Trace *b*: in = 0 mM Na^+, 0 mM Cl^-, 50 mM K^+, pH 7.1; out = 0 mM Na^+, 100 mM Cl^-, 50 mM K^+, 5 μM valinomycin, pH 7.1. Trace *c*: in = 0 mM Na^+, 0 mM Cl^-, 0 mM K^+; out = 0 mM Na^+, 100 mM Cl^-, 0 mM K^+. Solutions were maintained isotonic and at the desired initial pH as specified in Materials and Methods. All the external solutions contained 6 μM acridine orange. ↑ indicates addition of Na-gluconate alone (final concentration = 28 mM; traces *a'*, *b'*) or preceded by addition of monensin (final concentration = 5 μM, trace *c'*). Each trace has been chosen to be representative of the average time course of each experiment

under these more basic conditions, vesicles from the same preparation (i.e., also preloaded with K^+) were injected in a K^+-and Cl^--free solution containing nigericin (20 μM). A rapid and marked initial quenching was observed followed by fluorescence recovery (Fig. 5, traces *b* and *b'*: $\Delta F = 46.5 \pm 3.5\%$;

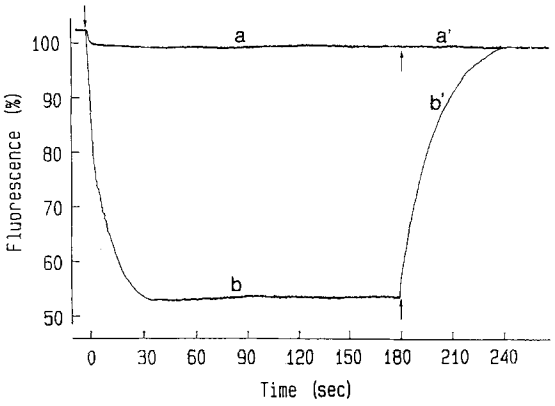

Fig. 5. Intravesicular pH variations dependent on an inwardly directed $[Cl^-]$ gradient when pH was initially 8 on both sides. ↓ indicates injection of BBMV. Trace *a*: in = 0 mM Na^+, 0 mM Cl^-, 50 mM K^+, pH 8; out = 0 mM Na^+, 100 mM Cl^-, 50 mM K^+, 5 μM valinomycin, pH 8; trace *b*: in, the same as for trace *a*; out = 0 mM Na^+, K^+, Cl^-, 20 μM nigericin, pH 8. Solutions were maintained isotonic and at the desired pH as described in Materials and Methods. All the external solutions contained 6 μM acridine orange. ↑ indicates addition of Na-gluconate (final concentration 28 mM, trace *a'*) or K-gluconate (final concentration 50 mM, trace *b'*). Each trace has been chosen to be representative of the average time course of each experiment

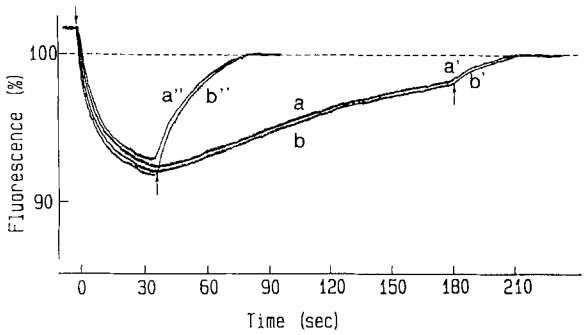

Fig. 6. Transvesicular ΔpH due to an outwardly directed $[Na^+]$ and $[Cl^-]$ gradient. ↓ indicates injection of BBMV. Trace *a*: in = 100 mM Na^+ and Cl^-, 50 mM K^+, pH 7.1; out = 0 mM Na^+ and Cl^-, 50 mM K^+, 5 μM valinomycin, pH 7.1; trace *b*: as for trace *a* but with 10^{-4} M furosemide in the external medium. Solutions were maintained isotonic and at the desired pH as described in Materials and Methods. All the external solutions contained 6 μM acridine orange. ↑ indicates addition of Na-gluconate (final concentration = 28 mM, traces *a'*, *b'*, *a''*, and *b''*). Each trace has been chosen to be representative of the average time course of each experiment

n = 4; *see also* Table 1) on K-gluconate addition (final concentration = 50 mM).

Transvesicular ΔpH Due to Outwardly Directed Na^+ and Cl^- Concentration Gradients

The aforementioned results indicate that a $[Cl^-]$ gradient alone does not cause changes in fluorescence by electrically silent transport. The possibility of an electroneutral coupling of Cl^- fluxes with Na^+ movements and pH changes was studied. When under short-circuit conditions both $[Na^+]$ and $[Cl^-]$ gradients were present ($Na_i = Cl_i = 100$ mM; $Na_o = Cl_o = 0$ mM), vesicle injection caused a fluorescence quenching that attained its maximum in 20 to 30 sec and then quickly decreased (Fig. 6, trace *a*). Fluorescence recovery following Na^+ addition at 3 min (Fig. 6, trace *a'*; $\Delta F = 1.50 \pm 0.5\%$; $n = 11$; different from zero, $P < 0.01$) was markedly reduced compared to the values obtained at 30 sec (Fig. 6, trace *a''*; $\Delta F = 7.5 \pm 0.9\%$; $n = 11$). This is a reduction of about 80% ($P < 0.01$). Na^+-dependent fluorescence recovery at 3 min under such conditions was also decreased compared to that obtained when only a $[Na^+]$ gradient was present (*see* Table 1 and second section of the Results). The differences were significant: $P < 0.05$ by paired analysis performed on samples from the same preparation loaded with different solutions, and $P < 0.01$ by the unpaired *t* test. To rule out the possibility that our results were due to the activation of a Cl^-/OH^- (HCO_3^-) exchange, we repeated the experiments in the same conditions and in the presence of an exchange inhibitor in the external solution. Cl^-/HCO_3^- exchange can be inhibited by furosemide (Cremaschi et al., 1987*a*) and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate (SITS) but the latter markedly reduced fluorescence when added to a test solution containing AO without vesicles, whereas the former had no effect. The presence of furosemide (10^{-4} M) had no effect on fluorescence changes with respect to controls (Fig. 6, trace *b*; 30 sec, trace *b''*: $\Delta F = 7.7 \pm 1.9\%$; 3 min, trace *b'*: $\Delta F = 1.6 \pm 1.0\%$; $n = 4$).

We then sought to establish whether the outwardly directed $[Cl^-]$ and $[Na^+]$ gradients or the presence of Cl^- by itself influenced $[Na^+]$ gradient-dependent pH changes. Vesicles were preloaded with $Na^+ = Cl^- = 100$ mM, $K^+ = 50$ mM, and were injected in a medium containing 100 mM Cl^-, 0 mM Na^+, 50 mM K^+ + 5 μM valinomycin.

Initial fluorescence quenching was slightly greater with respect to the condition when no Cl^- was outside and its dissipation was largely reduced (Fig. 7, trace *a*; *see also* Table 1). The Na^+-dependent fluorescence recovery at 3 min (Fig. 7, trace *a'*) was therefore significantly different ($P < 0.01$; $\Delta F = 9.3 \pm 2.0\%$; $n = 5$) from that obtained when the same vesicles were injected into a Cl^--free solution

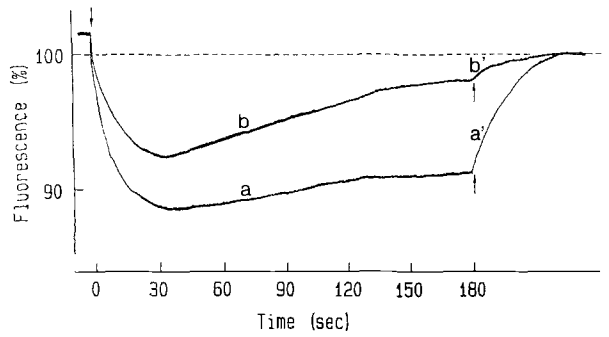

Fig. 7. Intravesicular pH variations dependent on an outwardly directed $[Na^+]$ gradient in the presence of equal intra- and extravesicular Cl^- concentration (trace *a*): trace *b*: presence of bumetanide (10^{-5} M) in the external medium together with an outwardly directed $[Cl^-]$ gradient. ↓ indicates injection of BBMV. Trace *a*: in = 100 mM Na^+ and Cl^-, 50 mM K^+, pH 7.1; out = 0 mM Na^+, 100 mM Cl^-, 50 mM K^+, 5 μM valinomycin, pH 7.1. Trace *b*: in, the same as for trace *a*; out = 0 mM Na^+, Cl^-, 50 mM K^+, 5 μM valinomycin, 10^{-5} M bumetanide. Solutions were maintained isotonic and at the desired pH as described in Materials and Methods. All the external solutions contained 6 μM acridine orange. ↑ indicates addition of Na-gluconate (final concentration 28 mM, traces *a'*, *b'*). Each trace has been chosen to be representative of the average time course of each experiment

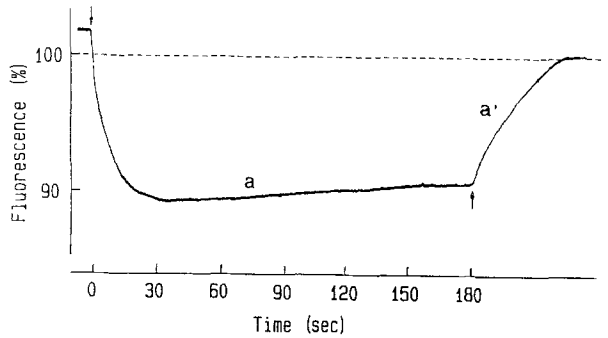

Fig. 8. Transvesicular ΔpH due to an outwardly directed $[Na^+]$ and $[Cl^-]$ gradient in the presence of hydrochlorothiazide (5×10^{-4} M). ↓ indicates injection of BBMV. In = 100 mM Na^+ and Cl^-, 50 mM K^+, pH 7.1; out = 0 mM Na^+, 100 mM Cl^-, 50 mM K^+, 5 μM valinomycin, pH 7.1. ↑ indicates addition of Na-gluconate (final concentration 28 mM). The trace has been chosen to be representative of the average time course of each experiment

($\Delta F = 1.3 \pm 0.8\%$; $n = 5$). Conversely, the overall trace and the quantitative parameters taken as reference were not significantly different from those obtained with a Na^+ gradient in the absence of vesicular and extravesicular Cl^-. Considering the presence of K^+, previous data (i.e., the rapid dissipation of fluorescence quenching) could be ascribed to Na^+-K^+-$2Cl^-$ cotransport, which would extrude Na^+ and hence dissipate its gradient. To test this possibility, vesicles were preloaded with $Na^+ = Cl^- = 100$ mM, $K^+ = 50$ mM, and injected into a solution containing $Na^+ = Cl^- = 0$ mM, $K^+ = 50$ mM, valinomycin = 5 μM and bumetanide = 10^{-5} M. The presence of bumetanide, which is known to be an inhibitor of Na^+-K^+-$2Cl^-$ cotransport in other cells (Palfrey, Feit & Greengard, 1980), did not modify the fluorescence changes elicited by vesicle injection (Fig. 7, trace *b*; trace *b'*: ΔF at 3 min = $1.9 \pm 0.8\%$; $n = 4$; *see also* Table 1).

Conversely, in experiments carried out using HCTZ (5×10^{-4} M), the fluorescence time course was similar to that obtained when only a $[Na^+]$ gradient was present (Fig. 8). In fact, the decrease in quenching elicited by Na^+ addition to the external medium (3 min after vesicle injection) was not statistically different in the two experimental conditions (Fig. 8, trace *a'*: $\Delta F = 10.3 \pm 3.0\%$; $n = 4$; Fig. 1, trace *a'*: $\Delta F = 9.6 \pm 4.0\%$; $n = 5$), but was significantly different from that obtained when both $[Na^+]$ and $[Cl^-]$ gradients were present in the absence of HCTZ ($P < 0.01$; *see* Table 1).

Comparison between Vesicles Obtained by Separation with Ca^{2+} or Mg^{2+}

The various procedures used during the separative steps that produce the final vesicle preparation may result in different permeability patterns for different ions. The possibility of altering the membrane structure and introducing artificial conductances as well as that of varying the rate of many transport systems must always be considered, particularly when using Ca^{2+} (Sabolic & Burckhardt, 1984). Our separation method also allows addition of Mg^{2+} (Mg^{2+} treatment) instead of Ca^{2+} (Ca^{2+} treatment) to the homogenate before the first centrifugation.

On this basis, we repeated the most significant experiments among those already described, utilizing vesicles prepared by Mg^{2+} treatment. The patterns of fluorescence changes obtained in different experimental conditions after vesicle injection closely resembled those observed with vesicles from Ca^{2+} treatment. Table 2 shows the Na^+-dependent fluorescence recovery 3 min after vesicle injection in various experimental conditions. Also in this case a $[Na^+]$ gradient induced an intravesicular acidification ($\Delta F = 29.8 \pm 5.3\%$; $n = 5$). Short circuiting the transmembrane electric potential difference reduced but did not abolish this acidification ($\Delta F = 10.6 \pm 2.2\%$; $n = 7$). An inwardly directed $[Cl^-]$ gradient did not cause detectable intravesicular acidification under short-circuit conditions. When outwardly directed $[Na^+]$ and $[Cl^-]$

Table 2. Comparison between vesicles prepared with Ca^{2+} or Mg^{2+} treatment referred to Na^+ dependent fluorescence recovery ($\Delta F\%$) obtained 3 min after vesicle addition

Experimental conditions	$\Delta F\%$		P^* (Mg^{2+} *vs.* Ca^{2+})
	Mg^{2+}	Ca^{2+}	
$[Na^+]$ gradient (A)	29.8 ± 5.3 (5)	27.9 ± 4.8 (4)	NS
$[Na^+]$ gradient (B) (PD = 0)	10.6 ± 2.2 (7)	9.6 ± 4.0 (5)	NS
$[Cl^-]$ gradient (C) (PD = 0)	0.2 ± 0.2 (3)	0.1 ± 0.1 (4)	NS
$[Na^+]$, $[Cl^-]$ gradient (D) (PD = 0)	2.0 ± 1.1 (5)	1.5 ± 0.5 (11)	NS
	A *vs.* B, $P < 0.05$; B *vs.* D, $P < 0.05$;	A *vs.* B, $P < 0.05$; B *vs.* D, $P < 0.01$;	

* P = statistical probability evaluated by Student's t test.

gradients (always under short-circuit conditions) were present, the Na^+-dependent fluorescence recovery 3 min after vesicle addition was reduced to very low values (ΔF = 2.0 ± 1.1%; n = 5). Similarly, using vesicles obtained by separation with Ca^{2+} or Mg^{2+}, there was no difference in the measures of the fluorescence changes ($\Delta F\%$ sec^{-1}) induced by the electrically neutral Na^+/H^+ exchange, in the presence and in the absence of amiloride at all $[Na^+]$ tested (*see* Table 3).

Discussion

Reliability and Advantages of the Method

Owing to the small size of the rabbit gallbladder, we could rarely obtain more than 30 μl (protein concentration = 6.5 mg/ml) of vesicle suspension even starting with 100 gallbladders. If measurements had to be performed with a standard fluorimeter, a single test would have required about 10 to 20 μl of vesicle suspension. Also, uptake measurements with ^{36}Cl or ^{22}Na would have required similar quantities (100 to 200 μg of proteins) for each experimental point.

The unconventional use of a microfluorimeter allowed us to bypass this problem. At the same time, the good correlation between fluorescence and ΔpH variations in the range used guaranteed the reliability of the method. Preliminary data (*not shown*) obtained on BBMV from rabbit intestine led to the same conclusion.

Na^+/H^+ Exchange

Coupling between the movement of H^+ and Na^+ ions is suggested by data obtained when a $[Na^+]$ gradient alone is imposed. Fluorescence quenching is observed even under short-circuit conditions although to a lesser extent, which indicates the presence of an electrically neutral exchange (Na^+/H^+).

The minor quenching with respect to the open-circuit condition indicates the presence of conductive fluxes (*see* conductance section) in parallel with the electroneutral exchange.

In the experiments with an imposed ΔpH and short-circuit conditions, the Na^+-dependent rate of dissipation of the initial fluorescence quenching confirmed the occurrence of a neutral Na^+/H^+ exchange exhibiting saturation kinetics (K_m = 6.3 mM). Such value is only slightly inferior to that found in intact *Necturus* gallbladder (11 mM) by Weinman and Reuss (1984) and in the same range as those reported for vesicles from intestinal (Hopfer & Liedtke, 1987) and renal epithelia (Ives et al., 1983; Kinsella & Aronson, 1980).

Amiloride is known to inhibit Na^+/H^+ exchange and to reach its maximal effect at concentrations greater than 10^{-3} M. At such concentrations, amiloride interferes with AO fluorescence (*data not shown*). Moreover, behaving as a weak base, it could alter the imposed pH gradient between the inside and outside of the vesicles, thus modifying the rate of spontaneous ΔpH dissipation (Dubinsky & Frizzell, 1983). However, 10^{-4} M amiloride, which is the concentration we used, had none of these effects, in agreement with observations in renal BBMV (Sabolic & Burckhardt, 1983*a*; Cassano et al., 1984).

Amiloride partially inhibited the Na^+/H^+ exchange at all tested Na^+ concentrations. The percentage of inhibition, which was inversely related to the Na^+ concentration, and the preliminary results obtained from kinetic experiments with the inhibitor, which showed an increase in the $K_{max,app}$ while the $V_{max,app}$ was not changed, are in agreement with a possible competition for a common binding site

Table 3. Rates of the electrically neutral Na^+/H^+ exchange, with or without amiloride, in vesicles obtained from Ca^{2+} or Mg^{2+} separation

Experimental conditions	$\Delta F\%$ sec^{-1}		P*
	Mg^{2+}	Ca^{2+}	
(A) Control (Na^+ = 28 mM)	1.8 ± 0.4 (5)	1.6 ± 0.2 (5)	NS
(B) Amiloride (10^{-4} M) (Na^+ = 28 mM)	1.3 ± 0.3 (5)	1.0 ± 0.2 (5)	NS
	A-B, $P < 0.02$	A-B, $P < 0.01$	
(C) Control (Na^+ = 4.7 mM)	0.6 ± 0.1 (5)	0.6 ± 0.1 (5)	NS
(D) Amiloride (10^{-4} M) (Na^+ = 4.7 mM)	0.3 ± 0.1 (5)	0.3 ± 0.1 (5)	NS
	C-D, $P < 0.01$	C-D, $P < 0.01$	

* P = statistical probability evaluated by Student's t test.

(Kinsella & Aronson, 1980; Knickelbein, Aronson & Dobbins, 1982).

Cl^-/OH^- Exchange

Our data suggest that Cl^- movements may be coupled to H^+ or OH^- only through an electrical pathway (*see* conductance section). Experiments performed at a neutral pH and at alkaline pH (to favor Cl^-/OH^- activation), did not provide any evidence for the presence of a neutral Cl^- coupling with the movements of H^+ or OH^-. However, the same vesicles were normally functional, since they could maintain a pH gradient, as shown by experiments utilizing nigericin in the presence of a $[K^+]$ gradient.

Lack of evidence for this neutral transport could be due to experimental conditions that lead to an underestimation of proton intravesicular accumulation. For instance, short circuiting was achieved by utilizing the same K^+ concentration inside and outside the vesicles and by adding valinomycin. However, inadequacy of preloading could result in a lower intravesicular K^+ concentration than expected theoretically. As a consequence, the inwardly directed $[K^+]$ gradient might give rise to an electric potential difference that would induce a H^+ efflux through the membrane. In fact, inadequacy of K^+ preloading in vesicles has already been observed (Lipkowitz & Abramson, 1987). We tried to overcome this problem by prolonged washing of the preparation in the same preloading solution before the final resuspension. However, even in the presence of the same concentrations of K^+ inside and outside the vesicles, the formation of a pH gradient could be prevented by a short-circuit mechanism involving a H^+/K^+ exchange, as suggested for renal epithelium vesicles (Binder & Murer, 1986). It is difficult to postulate the presence of such a mechanism in rabbit gallbladder unless it acts at a very slow rate. Considering intracellular K^+ activity (a_{iK} = 54–80 mM; Gunter-Smith & Shultz, 1982; Cremaschi et al., 1983) and assuming a pH difference between the inside and outside similar to that observed in *Necturus* (−0.2 pH units; Weinman & Reuss, 1982), electrochemical potentials for H^+ and K^+ favor proton absorption and K^+ secretion. However, K^+ secretion is negligible (Gunter-Smith & Schultz, 1982). Moreover, it is not easy to explain the presence on the membrane of a K^+-dependent mechanism that absorbs protons while the Na^+/H^+ exchange accomplishes H^+ secretion, considering that intracellular pH should favor a strict functional coupling of the Na^+/H^+ and Cl^-/HCO_3^- exchanges. Lack of evidence for a Cl^-/OH^- exchange could be due to a high conductance for H^+ that possibly parallels a low neutral countertransport rate, as hypothesized for renal BBMV (Chen, Illsey & Verkman, 1988). However, a high conductance for H^+ is not in agreement with the slow dissipation rate observed for a preset pH gradient (about 5% of initial ΔF in the first 30 sec under short-circuit conditions). Conversely, the slow rate of the exchange, together with the limitations due to the resolution of the technique, could explain why we did not detect Cl^-/OH^- exchange. This is in agreement with the data obtained in vesicles from other tissues using the AO method; $[Cl^-]$ gradients alone did not produce any detectable internal variation in pH (Sabolic & Burckhardt, 1983*b*; Cassano et al., 1984; Seifter, Knickelbein & Aronson, 1984; Ives, Chen & Verkman, 1986). The low transport rate could be due to the fact that, under physiological conditions, bicarbonate and not OH^- might be exchanged for Cl^-. This is in accordance with the observation that in intact rabbit gall-

bladder Cl^-/HCO_3^- exchange inhibitors such as SITS or furosemide reduce the rate of Cl^- entry, thus attaining an inhibition level which never exceeds that obtained by eliminating HCO_3^- from the incubation medium, even in the presence of acetazolamide (Cremaschi et al., 1987*a*).

It should be noted that the lack of a fluorescence quenching dependent on Cl^- in the presence of valinomycin and K^+ (equivalent inside and outside concentrations) confirms that this situation allows an effective short circuit of the transmembrane electrical potential. Indeed, use of a similar procedure also resulted in a short-circuit condition in intestinal and renal BBMV (Sabolic & Burckhardt, 1983*b*, 1984; Cassano et al., 1984).

Na^+-Cl^- Symport

Under short-circuit conditions an inwardly directed $[Cl^-]$ gradient did not induce any measurable change in intravesicular pH, but when the same gradient was imposed together with a $[Na^+]$ gradient in the same direction, a significant acidification took place. However, the acidification was less than that obtained with a $[Na^+]$ gradient alone and, moreover, it was transient.

The AO method allows small pH variations to be detected, provided that the inside proton concentration is about 0.6 pH units higher than that outside (*see* first section of Results; Warnock et al., 1982). It follows that, if experimental conditions cause a marked intravesicular acidification, successive small pH variations due to a Cl^-/OH^- exchange could be detected. However, the rapid dissipation of acidification observed when outwardly directed $[Cl^-]$ and $[Na^+]$ gradients are both present is large and significant. It is difficult to conceive that it should be attributed to a Cl^-/OH^- exchange alone, whose effects are not detectable even at a more basic pH. This is also supported by the fact that furosemide (10^{-4} M), which completely inhibits Cl^-/HCO_3^- exchange in intact rabbit gallbladder (Cremaschi et al., 1987*a*), does not prevent the dissipation of acidification. One might suggest that Cl^- itself induces a larger Na^+ passive permeability since only with its presence is proton dissipation observed. However, this is not conceivable since only $[Cl^-]$ gradient and not Cl^- *per se* is efficient for this action.

Thus, it seems reasonable to conclude that the $[Cl^-]$ gradient induces a progressive reduction in the $[Na^+]$ gradient, which results in a minor and transient proton accumulation.

All data suggest the existence of a Na^+-Cl^- symport, possibly K^+ independent since bumetanide had no effect. Bumetanide, also in intact rabbit gallbladder, does not modify the unidirectional Cl^- influx across the apical membrane, or the intracellular activities of Na^+ and Cl^- (Cremaschi et al., 1987*b*). In contrast, in preliminary experiments with HCTZ (5×10^{-5} M) we observed a significant reduction in the rapid proton dissipation usually observed in the presence of a $[Na^+]$ and $[Cl^-]$ gradient. Similar features (insensitivity to furosemide and bumetanide and dependence on HCTZ) have been described for the Na^+-Cl^- symport of the urinary bladder of winter flounder (Duffey & Frizzell, 1984; Stokes, 1984).

The fact that the maximum fluorescence quenching observed in the presence of a $[Na^+]$, $[Cl^-]$ gradient was only about 25% less than that observed when a $[Na^+]$ gradient alone was present (PD = 0), does not mean that the Na^+ transport due to the Na^+-Cl^- symport is minimal compared to that due to the Na^+/H^+ exchanger. Some observations are necessary to compare the amount of Na^+ moved by the Na^+/H^+ exchanger and the Na^+-Cl^- symport. The maximum fluorescence quenching measured in the presence of a $[Na^+]$ gradient and the calculated pH change ($\Delta pH = 0.9$—*see* equation in paragraph 1 of Results) indicate that the amount of H^+ exchanged, taking into account the internal buffer concentration (1 mM) and the external pH (7.1), cannot exceed the 1 mM multiplied by the total intravesicular volume. If protons are exchanged only through the Na^+/H^+ exchanger, whose stoichiometry is 1 : 1, intravesicular $[Na^+]$ variation due to this mechanism is the same. Soon after vesicle injection there is a transient acidification, which indicates that the $[H^+]_{in}/[H^+]_{out}$ ratio is increasing while the $[Na^+]_{in}/[Na^+]_{out}$ ratio[1] is decreasing. If the Na^+/H^+ exchanger is the only pathway of movement for the two ions, maximum fluorescence quenching is reached at equilibrium, when the $[Na^+]_{in}/[Na^+]_{out}$ ratio is equal to the $[H^+]_{in}/[H^+]_{out}$ ratio. If Na^+ can leave vesicles by another mechanism besides the Na^+/H^+ exchanger, equality of the ratios, once it is reached (soon before H^+ flux is reversed), is transient and cannot be maintained. Yet, the maximum fluorescence quenching represents again the point of equality of the two ratios (provided H^+ conductance is negligible; *see* conductance section of Discussion). In this case, it is clear that equality is reached at lower ratio values.

On the basis of the maximum fluorescence quenching measured in the presence of a $[Na^+]$ gradient and with electric PD = 0, utilizing the equation that correlates ΔpH and $\Delta F\%$ (*see* first section of

[1] Under our experimental conditions at the beginning $[Na^+]_{in}$ is 100 mM and $[Na^+]_{out}$ is 1.4 mM as a consequence of contamination of the extravesicular medium due to vesicle injection.

Results), it is possible to calculate an internal $[Na^+]$ = 11 mM.[2] With respect to this condition, the maximum fluorescence quenching observed in the presence of a $[Na^+]$ and $[Cl^-]$ gradient (PD = 0) is diminished by about three fluorescence units. Such a decrease in fluorescence quenching allows calculation of a new ratio between internal and external $[Na^+]$ that corresponds to a decrease in internal $[Na^+]$ of 2 mM (for the accuracy of this estimate *see* again considerations reported in footnote 2). This means that in the first 30 sec (i.e., the period needed to reach maximum fluorescence quenching in both cases), the Na^+-Cl^- symport causes an intravesicular $[Na^+]$ variation (about 2 mM) of at least the same entity as that due to the Na^+/H^+ exchanger (about 1 mM). However, the driving forces that act at the initial step on the two transport mechanisms are different and vary in a different way with time. As a consequence, an evaluation of the rate of the two transport mechanisms (Na^+/H^+ exchanger and Na^+-Cl^- symport) by calculating the amount of Na^+ transport during a relatively long period (30 sec) can only be indicative. On the other hand, trying to evaluate the different rates of transport by considering the initial rates of fluorescence variation that follow vesicle injection is not possible, owing to nonspecific quenching. Besides, one must consider that the strict test of initial rates, in that they are initial, is based on the fact that ionic intravesicular concentrations are not altered. In conclusion, a precise quantitative evaluation of the rates of the two mechanisms, at least in our conditions, seems quite difficult. One can only suggest that they are not markedly different from one another.

In intact epithelium, radiochemical experiments showed that half of the Cl^- entry across the apical membrane is supported by the Na^+-Cl^- symport and the other half by the Na^+/H^+ exchange (Cremaschi et al., 1987*a*).

Do the Symport and the Dual Exchange Operate Simultaneously?

In experiments where the existence of a Na^+-Cl^- symport was shown, our data were in agreement with a contemporary activity of the Na^+/H^+ exchanger. In fact, in the presence of a $[Na^+]$ and $[Cl^-]$ gradient we still observed an acidification, although it progressively diminished with time compared to that observed when only a $[Na^+]$ gradient was present. This means the simultaneous presence of an acidification mechanism (Na^+/H^+ exchange) and of a mechanism (Na^+-Cl^- symport) that reduces its rate by reducing the $[Na^+]$ gradient. Could the time course we observed alternatively be explained by a progressive inactivation of the Na^+/H^+ exchanger followed by the activation of the Na^+-Cl^- symport? The arguments contrary to this hypothesis are the following: (i) Since dissipation of the $[H^+]$ gradient through the conductive pathway is negligible (*see* the following section, "H^+, Na^+ and Cl^- Conductances"), only the Na^+/H^+ exchanger can be responsible for it when intravesicular $[Na^+]$ is reduced by Na^+-Cl^- cotransport. (ii) Addition of Na^+ to the external medium at 3 min should not result in a recovery of fluorescence.

Moreover, one should consider that it is difficult to justify the inactivation of the Na^+/H^+ exchange. (i) The cation exchanger works also in the presence of intravesicular $[Cl^-]$ (*see* experiments with equal $[Cl^-]$ inside and outside vesicles). (ii) Inside acidification by itself does not inactivate the exchanger (*see* experiments with $[Na^+]$ gradient). Our data in vesicles are in general agreement with those obtained in intact tissue. In the latter preparation, the Na^+-Cl^- symport was detectable only when the Na^+/H^+, Cl^-/HCO_3^- dual exchange was inhibited, so that it was impossible to establish whether the symport operated simultaneously with the double exchange or represented a homeostatic reaction of cells to the inhibition of the exchangers. Nevertheless, the fact that SITS and furosemide inhibited the exchange and revealed the symporter in about 1–2 sec suggested that the latter was already active before the addition of the inhibitor.

Parameters and modes of operation derived from experiments with vesicles do not necessarily apply to intact tissue. However, our results suggest that even in the latter the Na^+-Cl^- symport is probably always working and does not represent a homeostatic reaction induced by the experimental conditions following inhibition of the double exchange.

H^+, Na^+ and Cl^- Conductances

H^+ conductance is present although it appears to be minimal as shown by the slight decrease in fluorescence when a $[H^+]$ gradient is imposed in short-

[2] It is possible that this value is underestimated considering that the equation is derived from data obtained in different conditions (i.e., imposed ΔpH). Moreover, it is not certain that all the vesicles that can maintain an imposed ΔpH have the Na^+/H^+ exchanger. Yet, even admitting that the slope of the calibration curve might be 50% lower than the experimental one, the new equation ($\Delta F\% = 17\,\Delta pH - 10$) allows calculation of an intravesicular $[Na^+]$ of about 21 mM. The values of intravesicular $[Na^+]$ (11–21 mM), which are relatively low considering that preloading brings intravesicular $[Na^+]$ to 100 mM, are due to the Na^+ leakage relative to the Na^+ conductance and to the short-circuit conditions.

circuit conditions. There also seems to be present a Na^+ conductance, considering the results obtained with an outwardly directed Na^+ concentration gradient. The maximum fluorescence quenching (ΔF = 10.4%) obtained in short-circuit conditions and in the presence of a $[Na^+]$ gradient is markedly less than that predicted (ΔF = 42.9%) from the equation reported in the first section of Results, and the imposed ratio between internal and external Na^+ and assuming that only the Na^+/H^+ exchanger acts on $[Na^+]$. This finding suggests dissipation of the $[Na^+]$ gradient, i.e., Na^+ conductance could be appreciable (*see also* footnote 2). Such a hypothesis was confirmed when we compared fluorescence quenching obtained in the presence and in the absence of a short circuit. In the latter case, fluorescence quenching is greater, in agreement with the fact that the electric potential difference at least partially prevents dissipation of the Na^+ gradient.

As far as Cl^- conductance is concerned, we must refer to experiments with an imposed $[Cl^-]$ gradient (open-circuit conditions). The steady and high fluorescence quenching reached after vesicle injection suggests that the electrical potential difference reached is near to the equilibrium value. If this is the case, electrical potential difference and the related fluorescence variations are poorly influenced by Cl^- conductance.[3] In contrast, the fact that in short circuit no fluorescence quenching is observable makes it impossible to evaluate the amount of Cl^- entering the vesicles, since it is no longer prevented by an electrical potential difference. In this case, the absence of a detectable Cl^-/OH^- exchange prevents quantification of the Cl^- inside vesicles. In intact tissue the apical membrane of rabbit gallbladder epithelium has a high conductance for K^+, a slight conductance for Na^+, and no conductance for Cl^- (Cremaschi & Meyer, 1982). No data are available with respect to H^+ conductance. It is possible, therefore, that the Cl^- conductance revealed in the vesicles is too small to be measured with electrophysiologic methods used in the past or is dependent on the preparative procedure.

Possible Consequences of the Method of Vesicle Preparation

Results obtained with vesicles can vary according to the preparative method adopted. The choice of Ca^{2+} or Mg^{2+} in the initial preparation steps has been widely investigated. Some authors (Malathi et al., 1979; Yakymyshin, Walker & Thomson, 1982; Aubry, Merril & Proulx, 1986) maintain that vesicles from Mg^{2+} treatment are more contaminated than those from Ca^{2+} treatment. In our case, preparation with Mg^{2+} or Ca^{2+} gave similar enrichment values, although the latter gave a greater final yield than the former (29% instead of 15%; Bottà et al., 1987). In other epithelia, Ca^{2+} treatment has been shown to result in anomalous conductances for Na^+ or Cl^- and to reduce the efficiency of the Na^+/H^+ exchange (Sabolic & Burckhardt, 1984; Lipkowitz & Abramson, 1987) and the rate the Na^+-dependent glucose transport (Biber et al., 1981). The hypothesis that the effect of Ca^{2+} is due to activation of membrane phospholipases (Hauser et al., 1980) is not supported by analysis of the levels of free fatty acids and membrane phospholipids, which seem to remain unaltered even in the case of Ca^{2+} treatment (Aubry et al., 1986). A direct influence of Ca^{2+} on the vesicles also seems to be excluded (Sabolic & Burckhardt, 1984). In contrast, the isolation procedure itself seems to affect vesicle properties. The fact that we obtained similar results with vesicles from both treatments suggests no interference of Mg^{2+} or Ca^{2+} with the functional properties of the final preparation.

[3] Considering the maximum fluorescence quenching (ΔF = 41.5 ± 2.9%, n = 4, measured at 30 sec), on the basis of the calibration curve (*see* paragraph 1 of Results), one can calculate a ΔpH of about 2 units. However, taking into account also internal buffer concentration (1 mM), the amount of H^+ that entered the vesicles should be in the order of 1 mM multiplied by intravesicular total volume. Following electroneutrality of the solutions, this entry should be accompanied by an equivalent Cl^- entry that should bring internal $[Cl^-]$ to the order of 1 mM (considering that initial concentration is nominally zero). Let us consider that: (i) internal $[Cl^-]$ has reached a value of about 10^4 greater than the external $[H^+]$, (ii) internal $[H^+]$ (about 10^{-5} M) is negligible compared to external $[Cl^-]$. If Cl^- conductance is not totally negligible with respect to that of H^+ (which is reasonable considering the low H^+ conductance), the electrical potential difference is practically dependent only on $[Cl^-]$ (refer to the Hodgkin-Katz equation). This implies that electrical potential is very near to the equilibrium potential for Cl^-. A confirmation comes from the constancy of fluorescence, which is related to the electrical potential difference, after quenching has reached a maximum. The consequence is that Cl^- conductance could vary over a wide range without affecting electrical potential and consequently fluorescence variations.

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