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The Nature of the Neutral Na^+-Cl^--Coupled Entry at the Apical Membrane of Rabbit Gallbladder Epithelium: II. Na^+-Cl^- Symport Is Independent of K^+

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Summary. In the epithelium of rabbit gallbladder, in the nominal absence of bicarbonate, intracellular Cl^- activity is about 25 mM, about 4 times higher than intracellular Cl^- activity at the electrochemical equilibrium. It is essentially not affected by 10^{-4} M acetazolamide and 10^{-4} M 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (SITS) even during prolonged exposures; it falls to the equilibrium value by removal of Na^+ from the lumen without significant changes of the apical membrane potential difference. Both intracellular Cl^- and Na^+ activities are decreased by luminal treatment with 25 mM SCN^-; the initial rates of change are not significantly different. In addition, the initial rates of change of intracellular Cl^- activity are not significantly different upon Na^+ or Cl^- entry block by the appropriate reduction of the concentration of either ion in the luminal solution. Luminal K^+ removal or 10^{-5} M bumetanide do not affect intracellular Cl^- and Na^+ activities or Cl^- influx through the apical membrane. It is concluded that in the absence of bicarbonate NaCl entry is entirely due to a Na^+-Cl^- symport on a single carrier which, at least under the conditions tested, does not cotransport K^+.

Key Words gallbladder · Na^+-Cl^- symport · acetazolamide · SITS · SCN^- · bumetanide · K^+

Introduction

The conclusion of the previous paper (Cremaschi et al., 1987) was that two Na^+-Cl^- entry mechanisms exist (simultaneously or alternatively) at the apical membrane of rabbit gallbladder epithelium under steady-state conditions: the first is due to a Na^+/H^+, Cl^-/HCO_3^- double exchange, the second to a Na^+-Cl^- symport on a single carrier. The fact that the latter is not inhibited by furosemide even with high concentrations (10^{-3} M) suggests that it is not a Na^+-K^+-$2Cl^-$ symport similar to that found in the cortical thick ascending limb of Henle's loop (Greger & Schlatter, 1981) or in the flounder intestinal epithelium (Musch et al., 1982).

The aim of this paper is to investigate further the symport and its dependence on K^+ inasmuch as a Na^+-Cl^- symport independent of K^+, as originally proposed for epithelia (Nellans, Frizzell & Schultz, 1973), seems to be valid only in very few cases and sometimes with many uncertainties (Fossat & Lahlou, 1979; Duffey & Frizzell, 1984; Frizzell & Field, 1984; Stokes, 1984) and disputes (Larson & Spring, 1983; Alvo, Calamia & Eveloff, 1985).

In order to examine the symport without the overlap of the double exchange all the experiments were performed in the absence of bicarbonate in the bathing fluids.

Materials and Methods

Animals (New Zealand rabbits), preparation of gallbladder sheets and measurements of total Cl^- uptake and of the paracellular influx (45 sec) were as described in the previous paper (Cremaschi et al., 1987).

The setup for potential recordings was similar to that reported by Cremaschi et al. (1984) with the exception that the high impedance electrometer used was a dual-channel FD223 (World Precision Instruments Inc., New Haven, Conn.). The mounted tissue separated an upper mucosal from a lower serosal Lucite® chamber (window: 0.19 cm^2), both perfused (12 ml/min) with a bicarbonate-free saline; both chambers were kept inclined so to minimize fluid oscillations due to the peristaltic pump (Miniplus 2, Gilson, Villiers le Bel, France) as described by Zeuthen (1982). The time for renewal of the fluid on the epithelium was 4 sec.

General criteria for impalement validation were as previously reported (Cremaschi et al., 1984), but only micropunctures lasting several minutes were here selected as the effects of inhibitors or changes in ionic composition were followed generally during single impalements.

Measurement of K^+ Activity in the Extracellular Space

When K^+ was removed from the luminal bathing saline for uptake or for electrophysiological measurements, the K^+ activity was measured by a K^+-sensitive single-barrelled microelectrode placed externally near the apical membrane. Microelectrodes were constructed and calibrated as reported by Cremaschi and Meyer (1982). In the range of the calibration curve most important for our purposes the voltage change was: from 1 to 0.5 mM

Table 1. Effects of luminal acetazolamide and SITS on the apical membrane PD (V_m), intracellular Cl^- activity ($a_{i,Cl}$), and intracellular Cl^- at electrochemical equilibrium ($a^e_{i,Cl}$)[a]

	V_m	$a_{i,Cl}$	$a^e_{i,Cl}$
Control	−68.5 ± 2.4	25.7 ± 3.1	6.9 ± 0.7
	(8)	(8)	(8)
Acetazolamide	−69.3 ± 2.4	24.8 ± 3.1**	6.8 ± 0.6
(10^{-4} M)	(8)	(8)	(8)
Control	−76.1 ± 1.6	23.6 ± 2.2	5.0 ± 0.3
	(10)	(10)	(10)
SITS	−76.2 ± 1.6	23.6 ± 2.2	5.0 ± 0.3
(10^{-4} M)	(10)	(10)	(10)

[a] The values reported are steady-state values obtained by single impalements, with observations over more than 15 min. V_m is expressed in mV, activities in mM.

K^+ activity −7.2 mV, from 0.5 to 0.1 mM −6.2 mV, from 0.1 to nominally 0 mM −2.5 mV. Using $K^+_{K,NA}$ selectivity coefficients reported in the literature (Edelman et al., 1978; Cremaschi & Meyer, 1982), one can calculate with Nicolsky equation values similar to those above reported for the range between 1 and 0 mM K^+ activity. It is clear that measurements are reliable at least down to 0.1 mM K^+ activity.

Measurement of Intracellular Na^+ and Cl^- Activities

Na^+- or Cl^--sensitive theta microelectrodes (one conventional barrel and one ion-selective) with tips of 0.2 to 0.3 μm were constructed with the method reported by Meyer et al. (1985). Na^+-sensitive resin was purchased from Fluka AG, CH-9470, Buchs, Switzerland (10% ligand 1-ETH227, 89.5% 2-nitrophenyloctylether, 0.5% tetraphenylborate, wt/wt); Cl^--sensitive resin was the Corning Cl^--iron exchanger (477913). Na^+- and Cl^--sensitive microelectrode barrels were backfilled with 154 mM NaCl; the conventional barrels were backfilled with 500 mM KCl. Calibration curves for each Na^+- or Cl^--sensitive electrode were obtained using NaCl-KCl or NaCl-Nagluconate isosmotic test solutions with the sum of Na^+ and K^+ or of Cl^- and gluconate activities kept constant and equal to 112.4 meq (total concentration = 154 meq). When Na^+ or Cl^- activity of the calibration solution was changed from 112.4 to 11.2 meq, the voltage of the selective barrel changed by about 53 mV in both cases and that of the conventional barrel (variation of diffusion and tip potential altogether) was less than 1 mV. Resistances of Na^+- and Cl^--sensitive barrels were 6×10^{10} and 3×10^{10}, respectively. With the Ca^{2+} concentration of the phosphate-saline used Na^+ resin showed a small interference due to Ca^{2+}. The voltage variation introduced by the Ca^{2+} presence in the saline was subtracted to the measured values; the difference was used to determine activities on the calibration curve. Cl^- resin did not show dependence on phosphate and sulfate when these anions had the concentrations of the perfusion solutions used or those presumably present in the cell; conversely it exhibited a strong interference by SCN^- which was reduced, but not eliminated by inserting into the resin 0.5% tetraphenylborate (Ammann et al., 1983): $K_{Cl,SCN}$ was reduced from 10 to 4 by this procedure.

Table 2. Effects of Na^+ removal from luminal and serosal sides on the apical membrane PD (V_m), intracellular Cl^- activity ($a_{i,Cl}$), and intracellular Cl^- activity at electrochemical equilibrium ($a^e_{i,Cl}$)[a]

	V_m	$a_{i,Cl}$	$a^e_{i,Cl}$
Control	−66.1 ± 1.3	25.7 ± 1.5	7.5 ± 0.4
	(9)	(9)	(9)
Na^+-free	−67.0 ± 1.6	10.6 ± 1.7**	7.1 ± 0.4
	(8)	(8)	(8)

[a] The values reported are steady-state values obtained by multiple impalements. V_m is expressed in mV and activities in mM.

Salines

The phosphate-saline used contained (mM): Na^+ 145.3, K^+ 6.2, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 125.4, SO_4^{2-} 13.7, mannitol 12.5, phosphate 3.3, pH 7.4. Inhibitors were added directly to this solution since the change in osmolality was negligible with the exception of SCN^- (25 mM) which was substituted for SO_4^{2-} and mannitol. Na^+ was replaced with N-methyl-D-glucamine and K^+ with Na^+. In the experiments in which Ba^{2+} was used, it was substituted for Na^+; in this case the phosphate-buffer was replaced by 9 mM TrisOH/TrisCl (pH 7.4) and SO_4^{2-} by Cl^- so to avoid precipitations. Salines were bubbled with 100% O_2; incubation temperature was 27 ± 1°C. SITS and acetazolamide were purchased from Sigma Chemical Co. (St. Louis, Mo.); SCN^- from Carlo Erba-Farmitalia (Milano, Italy); bumetanide was a kind gift of Leo Pharmaceutical Products (DK-2750 Ballerups, Denmark).

Statistics

Results are presented in the Tables as means ± standard error of the mean with the number of experiments in parentheses. Statistical probability was analyzed with the *t*-test. When it was possible, the paired data analysis was used; asterisks denote values significantly different from controls (* = $P < 0.05$, ** = $P < 0.01$).

Results

Intracellular Cl^- Activity in the Absence of HCO_3^- from the Bathing Salines

The salines used did not contain HCO_3^- and were continuously bubbled with 100% O_2 in order to remove rapidly endogenously produced CO_2.

Intracellular Cl^- activity measured under these conditions was 24.9 ± 1.3 mM (27 exp.: Tables 1 and 2). The apical membrane potential difference (V_m) in the same cells was −70.5 ± 1.3 mV (cytoplasm negative), a value nearly equal and opposite to that of the basolateral membrane PD (V_s) within

Table 3. Effects of 25 mM SCN^- (in the lumen) on the apical membrane PD (V_m) and on intracellular Na^+ activity ($a_{i,Na}$)[a]

	Control	25 mM SCN^-			Control
Time (min)	0	1	2	3	6
V_m (mV)	−69.4 ± 1.2 (8)	−68.8 ± 1.5 (8)	−69 ± 1.7 (8)	−68.9 ± 1.7 (8)	−68.8 ± 1.2 (8)
$a_{i,Na}$ (mM)	11.9 ± 0.7 (8)	9.9 ± 0.7** (8)	9.5 ± 0.9** (8)	9.2 ± 0.9** (8)	12.3 ± 0.8 (8)

[a] During the entire period, 10^{-4} M SITS was present.

less than 0.5 mV. The calculated equilibrium of intracellular Cl^- activity was 6.4 ± 0.3 mM, i.e., about 4 times significantly lower than that actually measured.

Upon addition of acetazolamide (10^{-4} M) to the luminal solution during continuous impalements, V_m did not significantly change even after 15 min (paired data analysis with respect to control both before addition and after removal of the drug). In contrast, after 2 to 3 min a small, but significant decrease in intracellular Cl^- activity (less than 1 mM) was observed (Table 1).

Addition of SITS (10^{-4} M) in similar experiments caused no changes in either V_m or intracellular Cl^- activity even after 15 min (Table 1).

The lack of action of SITS and the minimal effect of acetazolamide indicate that the endogenous CO_2 production is such to maintain intracellular bicarbonate concentration to very low levels under our experimental conditions.

On the other hand, Cl^- is accumulated in the cell in a neutral way completely dependent on Na^+ (Table 2). Multiple impalements were made before removing Na^+ from both sides of the tissue. The transepithelial PD (V_{ms}) changed from −0.2 mV (serosa negative) to +0.5 mV after a long transient. Na^+ activity near the apical membrane (measured by a Na^+-sensitive microelectrode placed within 5 μm of the apical membrane) rapidly decreased from 145.3 to 0 mM. Additional impalements were made 15 min after Na^+ removal. Table 2 shows that Na^+ replacement by N-methyl-D-glucamine did not significantly affect V_m. This result is different from that previously obtained by Duffey et al. (1978) who found a depolarization of about 10 mV using choline as a substitute; it is known that choline reduces K^+ conductance, as demonstrated and discussed by Reuss and Finn (1975). In spite of the unchanged V_m, intracellular Cl^- activity decreased to 10.6 mM, a value which is significantly different from the value in the presence of Na^+ ($P < 0.01$), but which does not significantly differ from the equilibrium intracellular Cl^- activity (paired data analysis: $0.10 > P > 0.05$). The ratio between measured intracellular Cl^- activity and that at electrochemical equilibrium decreased significantly from 3.5 to 1.5. The latter value is not significantly different from 1.

This result is similar to that previously obtained by Duffey et al. (1978) in the presence of 21 mM HCO_3^- in the bathing salines; thus, both in the presence or absence of exogenous HCO_3^-, Cl^- is accumulated in the cell by an electroneutral, Na^+-dependent process, which persists even when endogenous HCO_3^- is nearly completely eliminated.

Effect of SCN^- on Intracellular Na^+ and Cl^- Activities

Impalements with double-barrel microelectrodes were also made in the absence of exogenous bicarbonate in both bathing fluids and in the presence of 10^{-4} M SITS on the luminal side. During the impalement the luminal solution was replaced by a second saline containing 25 mM SCN^- besides SITS. The results obtained are summarized in Table 3.

One can observe that intracellular Na^+ activity falls by about 2 mM reduction in the first minute of treatment ($P < 0.01$) and during the second and third minute continues to decrease slowly ($P < 0.05$), whereas V_m is not affected. The effect is completely reversible.

It is impossible to perform a similar experiment to assess the effect of SCN^- on intracellular Cl^- activity, because SCN^- strongly interferes with the Cl^--sensitive resin, even at low concentrations (*see* Materials and Methods). During an impalement with a double-barrel Cl^--sensitive microelectrode, SCN^- addition causes in a few seconds an increase in the apparent Cl^- chemical potential difference ($\Delta\mu_{Cl}$) across the apical membrane (i.e., a reduction in intracellular Cl^- activity as predicted, *see* part *A*

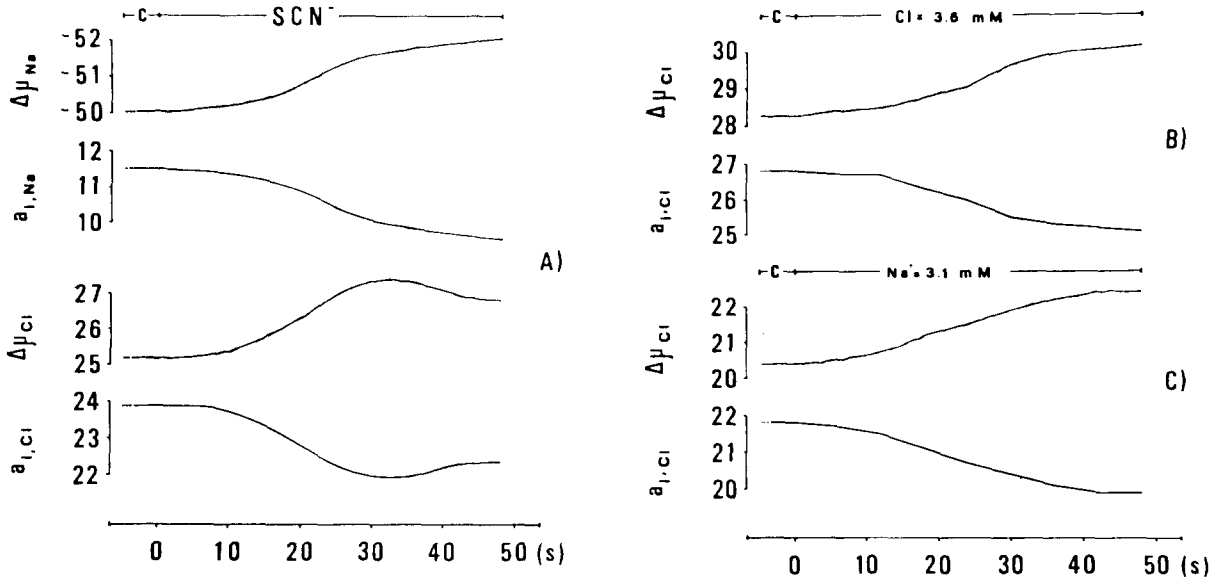

Fig. Changes in $\Delta\mu_{Cl}$, $\Delta\mu_{Na}$, $a_{i,Cl}$, $a_{i,Na}$ under different experimental conditions, during the first 50 sec of treatment. $\Delta\mu$ reported are expressed in mV and correspond to the apparent chemical potential difference measured by the microelectrode. The activities are given in mM. In *A*) 25 mM SCN^- was added to the lumen; in *B*) and *C*) luminal Cl^- or luminal Na^+ were reduced to 3.6 and 3.1 mM, respectively. Control periods are indicated as *C*

Table 4. Initial rates of change in intracellular Cl^- or Na^+ activities ($\Delta a_{i,Cl}/\Delta t$, $\Delta a_{i,Na}/\Delta t$) upon treatment with SCN^- or appropriate reduction of luminal Cl^- or Na^+ concentration

Luminal treatment	$\Delta a_{i,Cl}/\Delta t$ (mM/hr)	$\Delta a_{i,Na}/\Delta t$ (mM/hr)
25 mM SCN^-	576 ± 72 (5)	540 ± 120 (7)
3.6 mM Cl^-	480 ± 36 (7)	—
3.1 mM Na^+	510 ± 24 (7)	—

of the Figure). However, after about 35 sec the rate of increase in $\Delta\mu_{Cl}$ declines, then stops and $\Delta\mu_{Cl}$ starts to decrease. It is likely that SCN^- enters the cell and is sensed by the microelectrode. It is known that SCN^- is able to cross the apical membrane (Cremaschi, Hénin & Meyer, 1979). In conclusion, it is possible to determine only the initial rate of change of $\Delta\mu_{Cl}$ after treatment with SCN^-.

Initial Rates of Change in Intracellular Cl^- and Na^+ Activities

Part *A* of the Figure shows the initial changes in the apparent chemical potential difference of Cl^- ($\Delta\mu_{Cl}$) and Na^+ ($\Delta\mu_{Na}$) across the apical membrane upon addition of 25 mM SCN^- to the luminal saline. $\Delta\mu$ values are those directly sensed by the double-barrel selective microelectrode (the correction for the apical membrane potential difference is effected in real time). The corresponding cell activities calculated on this basis are also shown.

The change in the apparent $\Delta\mu_{Cl}$ and in $a_{i,Cl}$ was also determined by reducing nominally the luminal Cl^- activity to 3.6 mM or the luminal Na^+ activity to 3.1 mM (parts *B* and *C* of the Figure). These are the values predicted for equilibrium of the cotransport knowing that at the steady state the intracellular Na^+ activity is 11.9 mM (Table 3) and the intracellular Cl^- activity is 24.9 mM (Tables 1 and 2). Under these conditions, Cl^- or Na^+ entry by strict electroneutral cotransport through the luminal membrane should be blocked and the initial rate of change in cell ion activity should be a measure of the entry through the cotransport under steady-state conditions. However, this is true only if Na^+ and Cl^- fluxes are strictly coupled. Only in this case the initial rate of change for Cl^- activity will be approximately the same upon luminal Cl^- or Na^+ reduction (for a discussion of this point *see* Reuss, 1984).

The time-course of the change in $\Delta\mu$ and a_i for Cl^- or Na^+ is similar in the different experimental conditions. After a lag of time of about 4 to 6 sec (a) the change starts and continues with increasing velocity for 6 to 10 sec (b), then its rate becomes maximal and steady (c), and finally begins to decline at about 30 to 35 sec (d). Stage a) may be interpreted as the time of coarse renewal of the luminal saline (*see* Materials and Methods), stage b) as that of

Table 5. Effects of luminal K^+ removal on apical membrane potential (V_m) and intracellular Cl^- and Na^+ activities ($a_{i,Cl}$, $a_{i,Na}$)

	Control	K^+ = 0 mM			Control
Time (min)	0	1	2	3	6
V_m (mV)	−67.4 ± 1.6 (16)	−74.8 ± 1.7** (16)	−76.0 ± 1.8** (16)	−76.8 ± 1.8** (16)	−68.0 ± 1.7 (16)
$a_{i,Cl}$ (mM)	32.5 ± 4.0 (6)	33.2 ± 4 (6)	32.1 ± 4 (6)	31.8 ± 4 (6)	32.6 ± 4 (6)
$a_{i,Na}$ (mM)	11.3 ± 1.3 (10)	12.8 ± 1.3* (10)	13.0 ± 1.3** (10)	13.4 ± 1.3** (10)	10.8 ± 1.3 (10)

renewal of the saline in the unstirred layer close to the apical membrane, stage c) as the initial rate of change of the cell activity when the entry through the cotransport is actually blocked. In the case of treatment with SCN^- and measurement of apparent $\Delta\mu_{Cl}$ and $a_{i,Cl}$ a further stage (e) is present over which the apparent $\Delta\mu_{Cl}$ and $a_{i,Cl}$ are reduced and increased, respectively. This is clearly due to the interference of SCN^- (entering the cell) with the Cl^--sensitive resin.

The initial rates of change measured during stage c) are summarized in Table 4. Upon SCN^- treatment the two rates of change in intracellular Cl^- and Na^+ activities are not significantly different between each other. Moreover, no significant difference is present among the initial rates of change in intracellular Cl^- activity upon SCN^- treatment or reduction of the luminal Cl^- or Na^+ concentration.

K^+ Removal from the Bathing Fluids

K^+ activity in the unstirred layer close to the membrane was measured by a K^+-selective microelectrode whose tip was placed within 5 μm of the membrane. Under control conditions, K^+ activity in this layer was equal to that of the bulk solution (4.3 mM). When K^+ was removed from the bulk solution, its activity near the membrane was reduced to about 0.6 mM in 10 sec and below 0.1 mM in about 1 min. Similar results were obtained when 10 mM Ba^{2+} was added to the luminal saline in order to reduce the K^+ conductance in the apical membrane. At this concentration Ba^{2+} blocks K^+ channels in the apical membrane of *Necturus* (Reuss, Cheung & Grady, 1981) and rabbit gallbladder (*unpublished results from this laboratory*). The lack of additional effect of Ba^{2+} is expected since: i) under control conditions the value of the apical membrane PD is near the K^+ equilibrium PD so that K^+ leak toward the lumen is small (Gunter-Smith & Schultz, 1982; Cremaschi, Meyer & Rossetti, 1983); ii) when luminal K^+ is removed, V_m hyperpolarizes, but the K^+ conductance decreases (Hénin & Cremaschi, 1975; Garcia-Diaz, Nagel & Essig, 1983); iii) the rate of luminal superfusion is very high and facilitates K^+ removal in the unstirred layer.

Hence, we tested the effects of K^+ removal on intracellular Cl^- and Na^+ activities without treating the apical membrane with Ba^{2+}. The results obtained are shown in Table 5. The apical membrane hyperpolarized as expected ($P < 0.01$). However, no significant change in intracellular Cl^- activity was observed over 3 min after K^+ removal; intracellular Na^+ activity increased ($P < 0.01$), as expected from the small apical Na^+ conductance (Cremaschi & Meyer, 1982) and the hyperpolarization. The effects were completely reversible in 3 min.

The effects of K^+ removal on cellular Cl^- influx through the apical membrane were also tested using ^{36}Cl as a tracer. The luminal fluid was not changed continuously. The change in solution was abrupt, with five rapid washings with the K^+-free solution and subsequent addition of the tracer. In a sham uptake experiment we assessed the effectiveness of K^+ removal: K^+ activity measured by a selective microelectrode within the 5-μm-thick layer of fluid close to the membrane was 0.9 mM in 1.5 sec, 0.2 mM in 12 sec and virtually 0 mM in 18 sec after the five washings. Thus, the possible effect of K^+ removal on the uptake which is measured over 45 sec, should be evident.

The total and cellular Cl^- influxes measured in the presence and absence of luminal K^+ are shown in Table 6. The paracellular value used for the correction was 4.6 ± 0.3 μeq cm^{-2} hr^{-1} (32 exp.) and was measured as reported in the previous paper (Cremaschi et al., 1987). K^+ removal does not significantly affect cellular Cl^- influx.[1]

[1] The effects of K^+ removal on cellular influx were also tested in the presence of 10 mM Ba^{2+}. No significant variation was observed.

Table 6. Effects of luminal K^+ removal and of bumetanide on total and cellular Cl^- influx

Experimental condition (lumen)	Total Cl^- influx (μeq cm^{-2} hr^{-1})	Cellular Cl^- influx (μeq cm^{-2} hr^{-1})
Control	13.1 ± 0.7 (29)	8.5 ± 0.7 (29)
K^+-free	13.8 ± 1.4 (4)	9.2 ± 1.4 (4)
Bumetanide (10^{-5} M)	12.4 ± 1.1 (4)	7.8 ± 1.1 (4)

Bumetanide

The effects of 10^{-5} M bumetanide were tested both on the Cl^- and Na^+ activities. Table 6 shows that cellular Cl^- influx is not affected by drug addition to the luminal solution during the measuring time. Similarly, Table 7 demonstrates that the apical membrane PD and intracellular Na^+ and Cl^- activities are not significantly affected by the drug during a 3-min observation.

Discussion

The Na^+-Cl^- Symport in the Absence of Bicarbonate

In the previous paper (Cremaschi et al., 1987) we have shown that cellular Cl^- influx in the absence of bicarbonate from the bathing fluids is not affected by acetazolamide and SITS, but exhibits an immediate dependence on Na^+ and is inhibited by SCN^-.

The results presented here, concerning intracellular measurements of Cl^- activities, are in complete agreement. First, intracellular Cl^- activity is about 4 times greater than that expected from a distribution at electrochemical equilibrium. Second, it is minimally or insignificantly affected by acetazolamide and SITS even during prolonged exposures. Third, it is significantly and strongly reduced to the equilibrium value by luminal Na^+ removal without significant changes in the apical membrane PD. Fourth, it is affected by SCN^- even if it is technically impossible to determine exactly the extent of inhibition because of the interference of this anion with the Cl^--sensitive resin and because of its entry into the cell.

These results indicate the presence of a Na^+-CL^- cotransport which cannot be accounted for by a double exchange of Cl^-/HCO_3^- and Na^+/H^+.

Further evidence is provided by the observation that in the presence of SITS (to eliminate any trace of Cl^-/HCO_3^- exchange) SCN^- is also able to reduce rapidly the intracellular Na^+ activity and the initial rates of change in intracellular Na^+ and Cl^- activities are not significantly different. If the cotransport were actually a double exchange with indirect coupling of Cl^- and Na^+ influxes, the block of Cl^- entry should affect intracellular Cl^- activity rapidly, and Na^+ activity more slowly, as has been observed in *Necturus* gallbladder (Baerentsen, Giraldez & Zeuthen, 1983; Reuss, 1984). Moreover, the block of the cotransport by the appropriate reduction of luminal Cl^- concentration gives the same result obtained with SCN^- treatment. This eliminates any doubt on the validity of the initial rate measurement in the presence of SCN^- in the lumen. Finally, reducing Na^+ in the lumen causes a fall in $a_{i,Cl}$ also at the same initial rates measured after blocking Cl^- entry with SCN^- or reducing luminal Cl^-.

The Na^+ and Cl^- net influxes under steady-state conditions can be estimated from the initial rate of change in intracellular activities when Na^+ and Cl^- entry into the cell is abruptly stopped. It should correspond to the value of the flux measured transepithelially at the steady state (Reuss, 1984).

Na^+ and Cl^- transepithelial transport in the absence of bicarbonate in the bathing fluids is equal to about 7 μeq cm^{-2} hr^{-1} for both ions (Cremaschi et al., 1979). By assuming a mean correction factor of 3.8 for the real area of the epithelium due to foldings in respect to the apparent area of 1 cm^2 (Blom & Helander, 1977; Hénin et al., 1977), a cell height of 28 μm (Blom & Helander, 1977) and an activity coefficient of 0.73, one can calculate an initial rate of change in intracellular Na^+ or Cl^- activities of 482 mM hr^{-1}. This value is in good agreement with that experimentally determined. The same is equivalent to 8.0 mM min^{-1} a value which is larger than that reported by Reuss (1984), i.e. 4.8 mM min^{-1}; however, it is important to emphasize that transport rate, real/apparent area ratio, and cell height are different in the two epithelia compared.

In conclusion, the analysis with inhibitors of the system in steady state and the analysis of the transient effects of changes in ionic concentrations are both in accordance with the presence of a Na^+-Cl^- symport with a strict coupling of the two ions.

Is the Na^+-Cl^- Symport a Na^+-K^+-$2Cl^-$ Symport?

K^+ removal from the lumen has no effect on either cellular Cl^- influx or intracellular Cl^- and Na^+ ac-

Table 7. Effects of 10^{-5} M bumetanide (added to the luminal solution) on the apical membrane potential (V_m) and intracellular Cl^- and Na^+ activities ($a_{i,Cl}$, $a_{i,Na}$)

	Control	10^{-5} M bumetanide			Control
Time (min)	0	1	2	3	6
V_m (mV)	−64.5 ± 1.1 (19)	−64.7 ± 1.1 (19)	−64.5 ± 1.1 (19)	−64.6 ± 1.2 (19)	−64.4 ± 1.2 (19)
$a_{i,Cl}$ (mM)	27.5 ± 3.8 (12)	27.6 ± 3.8 (12)	27.6 ± 3.8 (12)	27.7 ± 3.8 (12)	28.5 ± 3.9 (12)
$a_{i,Na}$ (mM)	12.3 ± 0.6 (7)	12.4 ± 0.7 (7)	12.8 ± 0.8 (7)	12.9 ± 0.8 (7)	13.0 ± 0.9 (7)

tivities. However, the removal from the bulk solution did not immediately bring K^+ activity to 0 mM in the 5-μm-thick extracellular layer close to the membrane. This fact might produce misleading results, especially with the short time (45 sec) uptake measurements, even if in the latter case the abrupt change of the saline is much more efficient in removing K^+. Nevertheless some observations support the validity of the results. The K_m for K^+ of the Na^+-K^+-$2Cl^-$ cotransporter was found to range between about 6 and 25 mM in different tissues, when Na^+ and Cl^- were present at high concentrations (Rindlert, McRoberts & Saier, 1982; Palfrey & Rao, 1983). Thus, the measured decrease in K^+ activity is such that in a few seconds a large effect on Cl^- uptake should be exerted and in less than 20 sec the uptake should be stopped.

Bumetanide was also ineffective, in spite of the fact that this is the most potent inhibitor of Na^+-K^+-$2Cl^-$ symport (Palfrey, Feit & Greengard, 1980). This drug, at the dose used, should inhibit completely the cotransport. This result confirms what was previously observed with high concentrations of furosemide (Cremaschi et al., 1987).

On this basis, we conclude that no Na^+-K^+-$2Cl^-$ cotransport exists at the apical membrane. In addition, a model in which Na^+-K^+-$2Cl^-$ cotransport represents the entry mechanism for Cl^- does not fit with the analysis of Na^+, K^+ and Cl^- transepithelial fluxes. In the absence of bicarbonate, one measures a net transepithelial transport equal to 7.0 μeq cm^{-2} hr^{-1} for both Na^+ and Cl^- (Whitlock & Wheeler, 1967; Cremaschi et al., 1979); no net absorption of K^+ is observed (Wheeler, 1963; Gunter-Smith & Schultz, 1982). Since the entire Cl^- influx crosses the apical membrane through the cotransport (Cremaschi & Hénin, 1975; Cremaschi et al., 1979, 1983; Cremaschi & Meyer, 1982), a net apical flux of 7.0 μeq cm^{-2} hr^{-1} for Cl^-, 3.5 for Na^+ and 3.5 for K^+ should pass through this pathway in case the cotransport is a Na^+-K^+-$2Cl^-$ symport. Then, 3.5 μeq cm^{-2} hr^{-1} K^+ should recirculate back from the cell to the lumen through the apical K^+ channels (Hénin & Cremaschi, 1975; Cremaschi & Meyer, 1982) inasmuch as no net K^+ absorption occurs; moreover, another 3.5 μeq cm^{-2} hr^{-1} Na^+ should cross the epithelium (lumen to blood) through pathways parallel to the cotransport in order to account for the total net transepithelial transport of Na^+.

One can easily calculate the value of the apical K^+ flux, from cell to lumen, by knowing cell K^+ activity, apical K^+ conductance and apical membrane PD: it is 0.7 μeq cm^{-2} hr^{-1} (Cremaschi et al., 1983), a value much lower than that required by the hypothesis. Furthermore, the measured Na^+ fluxes are also inconsistent with the hypothesis. Three possible pathways, in principle, could accommodate altogether the 3.5 μeq cm^{-2} hr^{-1} residual flux of Na^+: i) the residual Na^+/H^+ exchange in the absence of exogenous bicarbonate, ii) the apical conductive pathway for Na^+, and iii) the paracellular route. The first and the second can account for about 0.2 μeq cm^{-2} hr^{-1} each (Cremaschi et al., 1979, 1983). The third component may be easily calculated from the shunt Na^+ conductance and the transepithelial PD (Cremaschi & Meyer, 1982; Cremaschi et al., 1983). Under these conditions, assuming that Na^+ activity in the lateral spaces is equal (instead of higher) to the luminal activity, the net absorption of Na^+ through this pathway is calculated to be 0.3 μeq cm^{-2} hr^{-1}. Hence, under the most favorable assumptions, the total absorption of Na^+ through pathways i), ii) and iii) (0.7 μeq cm^{-2} hr^{-1}) is much smaller than that required. The difference (2.8 μeq cm^{-2} hr^{-1}) cannot be accounted for by a solvent drag effect at the paracellular route; in fact, there is probably no difference in osmotic pressure across the junction, since the Na^+ reflection coefficient is low (van Os, de Jong & Slegers, 1974; van Os, Wiedner & Wright, 1979), and the net water

flux is nearly 100% transcellular (Wright, Smulders & Tormey, 1972; van Os & Slegers, 1973; van Os et al., 1974, 1979; Persson & Spring, 1982).

In conclusion, there is a large body of evidence against the presence of a Na^+-K^+-$2Cl^-$ cotransport.

Comparison with the Results of Other Authors

It is noteworthy that Diamond (1964) did not observe any effect on transepithelial water transport upon removal or addition of K^+ from or to the luminal solution in rabbit gallbladder. No effect of K^+ removal on NaCl entry through the apical membrane was found in *Necturus* gallbladder both by Larson and Spring (1983) and Reuss (1984) in spite of the fact that the former authors observed a large effect of bumetanide and the latter did not observe it. Only some experiments of Davis and Finn (1983) on *Necturus* gallbladder seem to indicate a K^+ dependence of NaCl entry, but only under conditions of cell swelling induced by ouabain. It is notable that Reuss (1984) did not observe any Na^+-Cl^- cotransport on a single carrier, but solely a Na^+/H^+, Cl^-/HCO_3^- double exchange so that the nondependence on K^+ is in the rationale of the findings. Conversely, Larson and Spring (1983) proposed Na^+-Cl^- cotransport on a single carrier as the sole mechanism of NaCl entry in the same animal species, apparently under the same conditions. It is very difficult to reconcile their results with those obtained by Reuss (1984), but it is difficult, too, to reconcile their data with ours as the Na^+-Cl^- cotransport on a single carrier that we observe in the absence of bicarbonate is independent of K^+ and insensitive to bumetanide; that is, it does not appear to share any feature with the Na^+-K^+-$2Cl^-$ cotransport. It has been previously discussed how our results can be easily reconciled with those of Reuss (Cremaschi et al., 1987).

Na^+-Cl^- cotransport of rabbit gallbladder appears quite similar to that recently demonstrated in the urinary bladder of winter flounder: insensitivity to amiloride, SITS, furosemide, bumetanide, acetazolamide and K^+ (Duffey & Frizzell, 1984; Stokes, 1984). It will be important to investigate the effects of hydrochlorothiazide, which is a potent inhibitor of the cotransport in the winter flounder (Duffey & Frizzell, 1984; Stokes, 1984). This inhibitor does not affect the Na^+-K^+-$2Cl^-$ symport of the thick ascending limb (Schlatter, Greger & Weidtke, 1983), but is a potent diuretic and saluretic, effective in the distal convolute tubule and medullary collecting duct (Kunau, Weller & Webb, 1975; Costanzo & Windhager, 1978; Hansen, Schilling & Wiederholt, 1981; Wilson, Honrath & Sonnenberg, 1983).

On the basis of our results, of the apparently conflicting reports on *Necturus* gallbladder, and of the data obtained in winter flounder urinary bladder, one may infer that under steady-state conditions at the apical membrane of the gallbladder: i) a double exchange exists which serves to Na^+ and Cl^- entry, ii) in species with low transport rates (*Necturus*) the double exchange is the predominant or even the sole working mechanism, also largely supported by endogenous CO_2 production, iii) in species with high transport rates (rabbit) the double exchange coexists with a cotransport on a single carrier which substitutes for it in the absence of exogenous bicarbonate or during specific inhibition, iv) the cotransport on a single carrier either is peculiar and similar to that of winter flounder urinary bladder or is a Na^+-K^+-$2Cl^-$ symport, however capable of being modulated by the different conditions so to be sensitive or insensitive to bumetanide and/or K^+. The latter observation might reconcile our findings and those of Reuss with those of Spring and co-workers if, even in a species with low transport rate, such as *Necturus*, Na^+-Cl^- symport can replace the double exchange under particular conditions which have not yet been investigated (e.g. pH, diet, etc.). It is interesting to point out that conflicting results have also been obtained in the thick ascending limb where some authors have found a Na^+-Cl^- cotransport sensitive to furosemide and K^+ (Greger & Schlatter, 1981; Hannafin, Kinne-Saffran & Kinne, 1983; Koenig, Ricapito & Kinne, 1983) and some other authors only to furosemide (Eveloff & Kinne, 1983; Alvo et al., 1985).

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