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The Nature of the Neutral Na^+-Cl^--Coupled Entry at the Apical Membrane of Rabbit Gallbladder Epithelium: I. Na^+/H^+, Cl^-/HCO_3^- Double Exchange and Na^+-Cl^- Symport

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Summary. Cl^- influx at the luminal border of the epithelium of rabbit gallbladder was measured by 45-sec exposures to $^{36}Cl^-$ and ^3H-sucrose (as extracellular marker). Its paracellular component was evaluated by the use of 25 mM SCN^- which immediately and completely inhibits Cl^- entry into the cell. Cellular influx was equal to 16.7 μeq cm^{-2} hr^{-1} and decreased to 8.5 μeq cm^{-2} hr^{-1} upon removal of HCO_3^- from the bathing media and by bubbling 100% O_2 for 45 min. When HCO_3^- was present, cellular influx was again about halved by the action of 10^{-4} M acetazolamide, 10^{-5} to 10^{-4} M furosemide, 10^{-5} to 10^{-4} M 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (SITS), 10^{-3} M amiloride. The effects of furosemide and SITS were tested at different concentrations of the inhibitor and with different exposure times: they were maximal at the concentrations reported above and nonadditive. In turn, the effects of amiloride and SITS were not additive. Acetazolamide reached its maximal action after an exposure of about 2 min. When exogenous HCO_3^- was absent, the residual cellular influx was insensitive to acetazolamide, furosemide and SITS. When exogenous HCO_3^- was present in the salines, Na^+ removal from the mucosal side caused a slow decline of cellular Cl^- influx; conversely, it immediately abolished cellular Cl^- influx in the absence of HCO_3^-. In conclusion, about 50% of cellular influx is sensitive to HCO_3^-, inhibitable by SCN^-, acetazolamide, furosemide, SITS and amiloride and furthermore slowly dependent on Na^+. The residual cellular influx is insensitive to bicarbonate, inhibitable by SCN^-, resistant to acetazolamide, furosemide, SITS and amiloride, and immediately dependent on Na^+. Thus, about 50% of apical membrane NaCl influx appears to result from a Na^+/H^+ and Cl^-/HCO_3^- exchange, whereas the residual influx seems to be due to Na^+-Cl^- cotranport on a single carrier. Whether both components are simultaneously present or the latter represents a cellular homeostatic counterreaction to the inhibition of the former is not clear.

Key Words gallbladder · Na^+-Cl^- cotransport · acetazolamide · furosemide · SITS · SCN^- · amiloride

Introduction

In prior years the nature of the neutral Na^+Cl^--coupled transport in epithelia has been greatly debated. The original model proposed for intestine (Nellans, Frizzell & Schultz, 1973) and gallbladder (Cremaschi, Hénin & Ferroni, 1974; Cremaschi & Hénin, 1975; Frizzell, Dugas & Schultz, 1975) was similar to that formulated by Crane for Na^+-glucose cotransport (Crane, 1962). However, a Na^+/H^+ and Cl^-/HCO_3^- double exchange was later revealed in many epithelia with a coupling between Na^+ and Cl^- due to cellular pH (Murer, Hopfer & Kinne, 1976; Liedtke & Hopfer, 1977; Heintze, Petersen & Wood, 1981; Petersen et al., 1981; Friedman & Andreoli, 1982; Liedtke & Hopfer, 1982*a*,*b*; Baerentsen, Giraldez & Zeuthen, 1983; Aronson & Seifter, 1984; Reuss, 1984). In other epithelia, Na^+-Cl^- cotransport was found to be dependent on K^+ and to be due in fact to a Na^+-K^+-$2Cl^-$ symport (Frizzell & Field, 1984; Greger, 1984); similar findings were also reported for nonepithelial cells (Geck et al., 1980; Aiton et al., 1981; Ellory et al., 1982; Russell, 1983). Simple NaCl cotransport seems to exist in some epithelia (Fossat & Lahlou, 1979; Duffey & Frizzell, 1984; Frizzell & Field, 1984; Stokes, 1984; Alvo, Calamia & Eveloff, 1985) and in others is disputed (Baerentsen et al., 1983; Reuss, 1984; Spring, 1984).

In a study in rabbit gallbladder (Cremaschi et al., 1983), a kinetics analysis of Cl^- uptake suggested the presence of both a double exchange and a symport on a single carrier when the epithelium was bathed on both sides by a saline which had the Cl^- concentration of the blood. When luminal Cl^- concentration was lower, a third, less certain transport system was revealed.

In this paper we confirm the presence of the two first systems by the use of presumably specific inhibitors such as thiocyanate, furosemide, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (SITS), amiloride and acetazolamide. Experiments were designed based on the knowledge that no Cl^- conductance exists at the apical membrane of rabbit gallbladder (Cremaschi & Hénin, 1975; Hénin & Cremaschi, 1975; Cremaschi & Meyer, 1982).

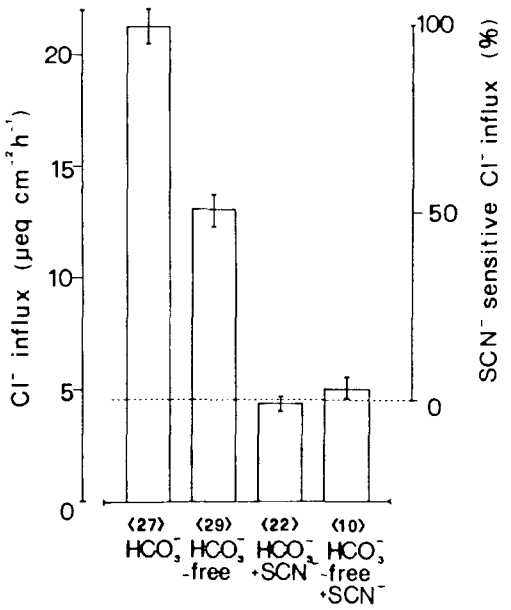

Fig. 1. Total Cl^- influx (ordinate on the left) and SCN^--sensitive Cl^- influx (ordinate on the right) from the lumen to the epithelium in the presence and in the absence of 25 mM HCO_3^- (45-min removal from both sides). SCN^- was only present during the measuring time (45 sec). The dashed line represents the weighted mean of the SCN^--insensitive Cl^- fluxes measured in the presence or absence of HCO_3^-

Moreover, total cellular Cl^- influx is Na^+-dependent (Cremaschi & Hénin, 1975; Cremaschi, Hénin & Meyer, 1979; Cremaschi et al., 1983) and inhibited by SCN^- (Cremaschi et al., 1979, 1983).

Materials and Methods

New Zealand rabbits were purchased by Azienda Agricola Bernasconi (Valmorea, Como, Italy). They were killed by a blow on the neck and gallbladders were excised and washed free of bile with Krebs-Henseleit solution.

Measurement of Cl^- Uptake

The method used was equal to that previously reported (Cremaschi et al., 1983) with the exception that the exposed tissue area was 0.60 cm^2. Briefly, gallbladders, opened flat, were mounted between two Lucite® chambers, luminal surface facing upwards; the tissue was supported by a nylon mesh. Fluid stirring in the luminal solution (1 ml) was obtained by bubbling the appropriate gas mixture; conversely the serosal chamber was continuously perfused with gassed saline moved by a peristaltic pump. After a 30-min preincubation period in Krebs-Henseleit solution, the tissue was allowed to equilibrate for 45 min again in Krebs-Henseleit solution or in a bicarbonate-free saline. Then it was washed 5 times on the luminal side with the test solution and finally exposed for 45 sec to the same saline to which ^3H-sucrose (10 μCi/ml) and $^{36}Cl^-$ (4 μCi/ml) were added. Variations in incubations on the luminal side will be described later. At the end of the experiment the tissue was processed as previously reported.

Under control conditions the tracer Cl^- uptakes were linear for at least 75 sec (Cremaschi et al., 1979); in the other tested conditions there was linearity for at least 45 sec.

Measurements of Na^+ Activity in the Luminal Unstirred Layer

When Na^+ was removed from the luminal bathing saline, the decrease in chemical activity was measured with a Na^+-selective single-barrelled microelectrode placed close to the apical membrane (within 5 μm of the surface). Five washings of the mucosa with the Na^+-free saline were performed to complete the removal. Na^+ activity decreased from 143 mM to virtually zero in 2 to 3 sec after the washings; the worst result obtained indicated a decrease to 1 mM. The control was continued for 1 to 2 min. Microelectrodes were constructed and calibrated as reported by Cremaschi et al. (1984). The highly Na^+-selective resin purchased from Fluka AG (ligand 1-ETH227) was not useful for these experiments due to the interference of Ca^{2+}, particularly at low Na^+ activity. Thus, we used the Na^+-sensitive resin purchased from WPI; it was affected by K^+ but not by Ca^{2+}. Calibration curves were prepared with isotonic salines in which Na^+ was gradually replaced by N-methyl-D-glucamine in the presence of constant concentrations of Ca^{2+} (2.5 mM), Mg^{2+} (1.2 mM) and K^+ (5.9 mM). When Na^+ activity was changed from 104.4 to 10.4 mM, the voltage changed by −46 mV; from 10 to 0 mM, the voltage change was −16 mV.

Instruments for voltage recordings were as described by Cremaschi et al. (1984).

Salines

Krebs-Henseleit solutions contained (mM): Na^+ 143, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 127.7, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2; pH 7.4. Bicarbonate replacement was with SO_4^{2-} and mannitol, Na^+ with N-methyl-D-glucamine. Bicarbonate-free solutions were buffered with 9 mM TrisOH/TrisCl (pH 7.4). Inhibitors were directly added to the saline, as they negligibly changed osmolality with the exception of SCN^-, which was substituted for Cl^- (in the solutions containing HCO_3^-) or SO_4^{2-} and mannitol (in the HCO_3^--free solutions). Salines were bubbled with 5% CO_2 and 95% O_2 when HCO_3^- was present and with 100% O_2 in its absence. Incubation temperature was 27 ± 1°C: this value was chosen since it seemed to give steadier transports.

Materials

Radioactive materials were purchased from the Radiochemical Centre (Amersham, Bucks, U.K.), Na^+-selective resins were from Fluka AG (CH-9470, Buchs, Switzerland) and from World Precision Instruments Inc. (New Haven, Conn.). SITS, acetazolamide and furosemide were from Sigma Chemical Co. (St. Louis, Mo.); SCN^- from Carlo Erba-Farmitalia (Milano, Italy); amiloride was a kind gift from Merck Sharp & Dohme (Rahway, N.J.).

Statistics

Results are generally presented as mean ± standard error of the mean; the number of experiments is reported in parentheses or is otherwise indicated; *t*-test was used for statistical comparisons.

Results

Bicarbonate Effects on Cl^- Uptake

In the presence of 25 mM bicarbonate in the bathing fluids total Cl^- influx (lumen to epithelium) was equal to 21.3 μeq cm^{-2} hr^{-1} as shown in the first bar in Fig. 1. When the tissue was bathed with bicarbonate-free saline on both sides, Cl^- influx decreased to 13.1 μeq cm^{-2} hr^{-1} ($P < 0.01$). In these experiments bicarbonate was absent for a 45-min preincubation time and during the 45-sec measuring time.

Third and fourth bars in Fig. 1 report influx values measured when 25 mM SCN^- was present in the lumen during the measuring time (45 sec), either with bicarbonate or with bicarbonate-free saline bathing both tissue sides during the preincubation (45 min) and the measuring (45 sec) time. A reduction of Cl^- influx to 4.4 and 5.1 μeq cm^{-2} hr^{-1} was obtained under these conditions ($P < 0.01$). These two values are not significantly different from each other so that bicarbonate does not appear to affect this SCN^--insensitive fraction. The mean of these two values (4.6 ± 0.3 μeq cm^{-2} hr^{-1}, 32 exp.) was used as reference for the SCN^--insensitive component.

The ordinate on the right in Fig. 1 indicates the SCN^--sensitive Cl^- influx. Bicarbonate removal reduces it to 50.9%. Since we showed that SCN^- abolishes all Cl^- entry into the cell (Cremaschi et al., 1979), the SCN^--sensitive and the SCN^--insensitive fractions of total Cl^- influx are cellular and paracellular, respectively. On these bases, we can now state that bicarbonate removal from the bathing solutions reduces the cellular component of the influx to about 50%.

Thiocyanate

Our previous conclusions on the action of 25 mM SCN^- were obtained with 45-min luminal exposures (Cremaschi et al., 1979). In the present studies we have tested both the time- and concentration-dependence of the inhibitor.

Figure 2 depicts the effects of different concentrations of SCN^- on total Cl^- influx (lumen to epithelium). SCN^- was added to the luminal solution,

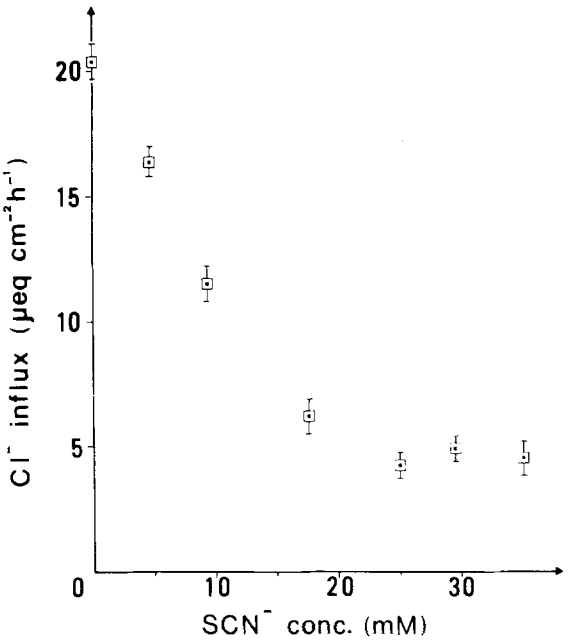

Fig. 2. Effect of luminal SCN^- concentration on total Cl^- influx (lumen to epithelium) in the presence of bicarbonate. SCN^- was present only during the measuring time (45 sec). Each point is the mean of seven experiments

only during the flux-measuring time (45 sec). Maximal effects are obtained at about 17 mM. The SCN^--insensitive fraction determined with this and higher concentrations is not statistically different from that reported in Fig. 1. The time of maximal action was tested by reducing the measuring period from 45 to 30 sec. If shorter periods are used, the experimental error rises. As expected (Cremaschi et al., 1979) the influx was not significantly different at 30 or 45 sec in the absence of SCN^- (20.6 ± 2.5 μeq cm^{-2} hr^{-1} at 30 sec; 21.0 ± 1.2 μeq cm^{-2} hr^{-1} at 45 sec; five determinations in each case). Comparatively similar results were obtained in its presence (25 mM) (4.4 ± 1.4 μeq cm^{-2} hr^{-1} at 30 sec; 4.0 ± 0.8 μeq cm^{-2} hr^{-1} at 45 sec; six determinations in each case). This indicates that SCN^- inhibition is exerted within a period much shorter than 30 sec.

The paracellular component of the influx (SCN^--insensitive fraction; *see* Introduction) was also measured under all experimental conditions subsequently tested. Only amiloride treatment significantly altered paracellular Cl^- influx from 4.6 ± 0.3 to 7.6 ± 0.7 μeq cm^{-2} hr^{-1} (32 and 18 experiments, respectively, $P < 0.01$). For this reason all paracellular fractions under different conditions of treatment were assumed equal to 4.6 μeq cm^{-2} hr^{-1}, with the exception of data in amiloride for which the specific paracellular value was subtracted.

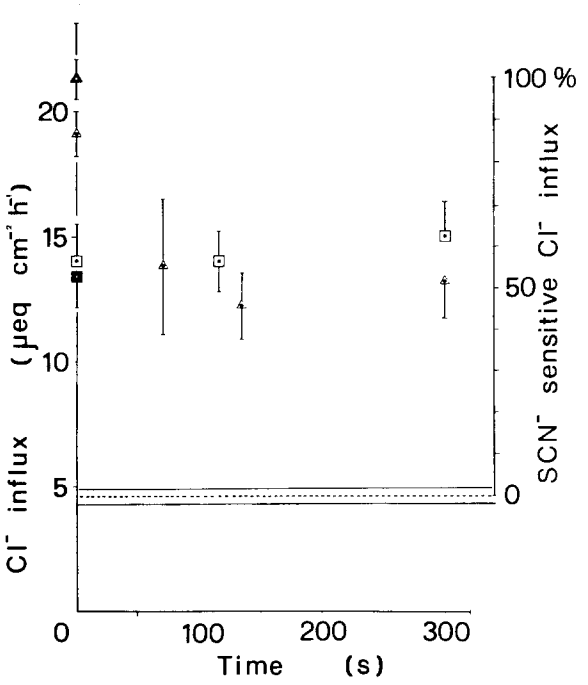

Fig. 3. Effects of 10^{-4} M acetazolamide on total Cl^- influx in the presence (△) or in the absence (□) of bicarbonate in the bathing fluids. The drug was added to the luminal side only during the measuring time (45 sec) and for the preincubation time indicated in the abscissa. The dashed line denotes the SCN^--insensitive paracellular component (mean ± SEM). ▲ control in the presence of bicarbonate. ■ control in the absence of bicarbonate. Each point is the mean of eight experiments

Acetazolamide

This drug was added to the luminal side at a 10^{-4} M concentration since under these conditions its action is specific on carbonic anhydrase (Maren, 1977) and both cytoplasmic and membrane-bound species of the enzyme are completely inhibited (Maren, 1980). The results are shown in Fig. 3.

When bicarbonate is present in both solutions, a significant inhibition from 21.3 to 13.8 μeq cm^{-2} hr^{-1} ($P < 0.01$) is reached only after a 75-sec preincubation with the drug, but less scattered effects seem to be achieved only after 135 sec.

When bicarbonate was removed from both bathing solutions for 45 min, no inhibition was obtained even after a 5-min preincubation with the drug. The influx remained at about 14 μeq cm^{-2} hr^{-1}, a value which is not significantly different from that obtained with bicarbonate and acetazolamide.

Subtracting the paracellular fraction, the cell Cl^- influx insensitive to acetazolamide (both with bicarbonate and bicarbonate-free salines) is about 55% of the cellular influx measured with bicarbonate salines bathing the tissue.

Furosemide

This drug was tested at different concentrations and was added to the luminal bicarbonate medium only during the measuring time as it is well known to operate very rapidly (1 to 2 sec) on the external side of cellular membranes (Greger, Schlatter & Lang, 1983). As shown in Fig. 4, maximal effects are reached at 10^{-5} to 10^{-4} M and are not increased by a 5-min 10^{-3} M preincubation.

When the paracellular influx is subtracted, the furosemide-insensitive influx turns out to be about 55% of the total cellular influx.

SITS

This drug was tested at different concentrations and added to the luminal bicarbonate medium during the measuring time. Results are reported in Fig. 5. Maximal effects are reached at 10^{-5} to 10^{-4} M and are not increased by a 5-min 10^{-3} M preincubation.

As with furosemide and acetazolamide, when the paracellular influx is subtracted, the SITS-insensitive influx is calculated to be about 50 to 55% of total cellular influx.

Furosemide and SITS

On the basis of the experiments reported above, one cannot discriminate whether furosemide, SITS and acetazolamide inhibit the same fraction of cellular influx or complementary fractions. To distinguish between these possibilities, the effects of SITS and furosemide on Cl^- influx were studied in the absence and in the presence of bicarbonate (Fig. 6).

When bicarbonate was nominally removed for 45 min, SITS (10^{-4} M) or furosemide (5×10^{-4} or 10^{-3} M) had no effect.

In the presence of bicarbonate, luminal addition of both SITS and furosemide (10^{-4} M) had a clear inhibitory effect (Fig. 6). The SITS- and furosemide-insensitive cellular influx turned out to be 9.3 μeq cm^{-2} hr^{-1}, a value which is not significantly different from that of SITS- (8.3 μeq cm^{-2} hr^{-1}) or furosemide- (9.2 μeq cm^{-2} hr^{-1}) insensitive influx.

Thus, the effects of SITS and furosemide are not additive. Both agents operate on the bicarbonate-dependent fraction of cell Cl^- influx.

Amiloride

This drug was added to the luminal bicarbonate-saline at a high concentration (10^{-3} M), sufficient to inhibit the Na^+/H^+ antiport. The preincubation pe-

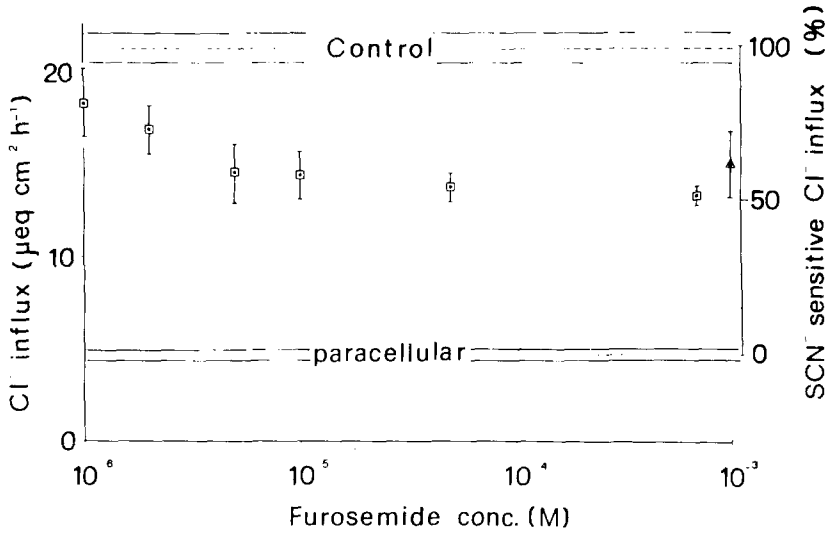

Fig. 4. Effects of luminal concentration of furosemide on total Cl^- influx in the presence of bicarbonate. The drug was added during the 45-sec measuring time (□) or for the 45-sec measuring time and for a 5-min preincubation period (△). The upper dashed line indicates the control (mean ± SEM) in the absence of furosemide; the lower dashed line denotes the SCN^--insensitive paracellular component (mean ± SEM). Each point is the mean of six experiments with the exception of the points corresponding to 5×10^{-5} and 7×10^{-4} M furosemide which are the mean of 10 and 29 experiments, respectively

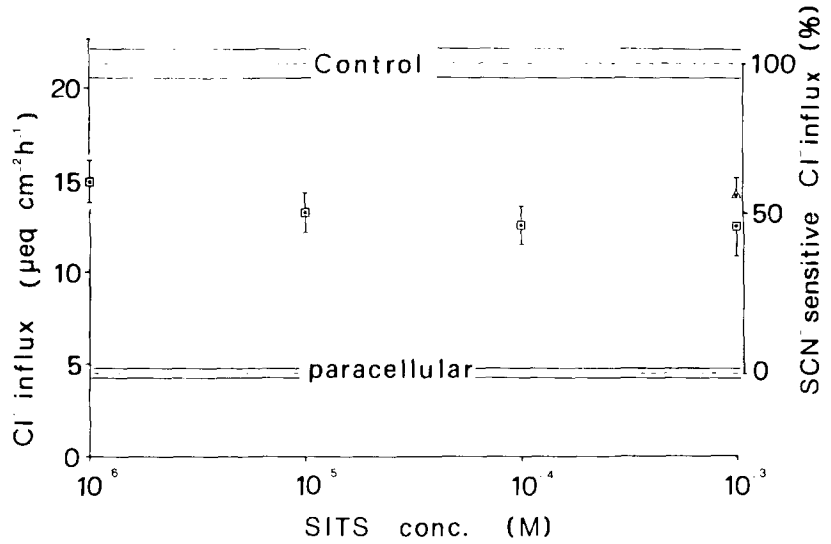

Fig. 5. Effects of luminal concentration of SITS on total Cl^- influx in the presence of bicarbonate. The drug was added during the 45-sec measuring time (□) or for the 45-sec measuring time and for a 5-min preincubation time (△). The upper dashed line indicates the control (mean ± SEM) in the absence of SITS; the lower dashed line indicates the SCN^--insensitive paracellular component (mean ± SEM). Each point is the mean of 11 experiments

riod was 5 min. Results are reported in Fig. 7, which shows a significant decrease of cellular Cl^- influx from 16.7 to 7.1 μeq cm^{-2} hr^{-1} ($P < 0.01$). If 10^{-3} M amiloride and 10^{-4} M SITS were added together, the result was not significantly different. This is evidence both that the two drugs have no additive effects and that amiloride has inhibited indirectly, but completely, a Cl^-/HCO_3^- exchanger (completely, in spite of the high Na^+ concentration of the saline).

The amiloride-insensitive fraction of cellular influx is 7.1 μeq cm^{-2} hr^{-1}, i.e., about 45% of control cellular influx.

Na^+ Removal

Na^+ was removed from the luminal side either in the presence of bicarbonate or in its absence from both sides.

If Na^+ was removed for 10 min in the presence of HCO_3^-, cellular Cl^- influx was abolished (Fig. 8). This result was expected as the entire Cl^- entry into the cell is known to be Na^+ dependent by an electrically neutral coupling (*see* Introduction). When Na^+ was removed only during the measuring time (45 sec), cellular influx decreased largely and significantly ($P < 0.01$), but was not eliminated (Fig. 8). This indicates that Cl^- influx, at least partially, if not totally, is slowly dependent on Na^+; however, it is not excluded that a fraction is immediately dependent on Na^+.

The experiment was repeated in the absence of HCO_3^- (45 min) and in the presence of 10^{-4} M SITS during the measuring time (45 sec) to be certain that the effects of exogenous and endogenous HCO_3^- were eliminated. Under these conditions, Na^+ removal during the measuring time completely abol-

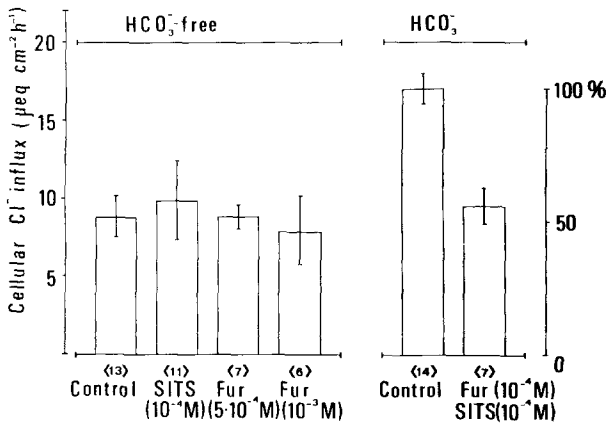

Fig. 6. Effects of luminal SITS or furosemide on cellular Cl^- influx in the absence of bicarbonate (left) and effects on cellular Cl^- influx of SITS and furosemide added simultaneously in the presence of bicarbonate (right). Drugs were only added during the 45-sec measuring time. The paracellular component (SCN^--sensitive fraction) has been already subtracted and does not appear in the Figure

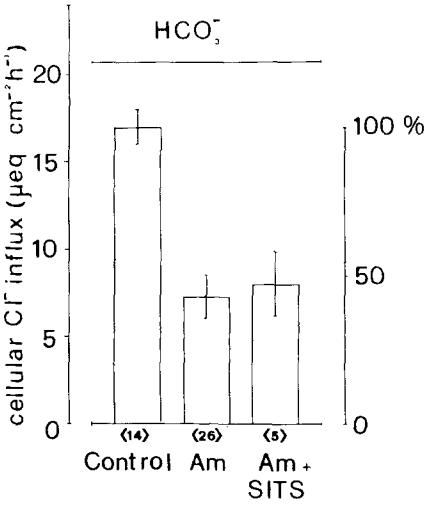

Fig. 7. Effects on cellular Cl^- influx of luminal 10^{-3} M amiloride or 10^{-3} M amiloride and 10^{-4} M SITS added together. The tissue was bathed with a bicarbonate-saline. Amiloride was added during the 45-sec measuring time and for a 5-min preincubation period. The paracellular (SCN^--insensitive) component has been already subtracted and does not appear in the Figure

ished cellular Cl^- influx (Fig. 8). This result indicates that when the bicarbonate-sensitive fractions are eliminated the residual cellular Cl^- influx is immediately dependent on Na^+.

These studies confirm the existence of fractions of Cl^- influx slowly and immediately Na^+ dependent (Cremaschi et al., 1983), under better controlled Na^+ removal and with the aid of SITS to eliminate the effects of HCO_3^-.

Discussion

The Nature of the Cotransport in Rabbit Gallbladder

In the apical membrane of the epithelial cells of rabbit gallbladder no Cl^- conductance seems to exist either in the presence or in the absence of HCO_3^- from the bathing fluids. Chloride entry into the cell is Na^+ dependent and inhibitable by SCN^- (Cremaschi & Hénin, 1975; Cremaschi et al., 1979),[1] an anion which competes for carrier-mediated Cl^- transport in many epithelial cells (Katz et al., 1982). In this paper we showed that SCN^- action is immediate and that the 25-mM concentration used in the past on this tissue (Sullivan & Berndt, 1973; Van Os, Michels & Slegers, 1976; Cremaschi et al., 1979) gives rapid maximal effects.

If the intact epithelium is used for Cl^- uptake instead of a membrane vesicle preparation, the paracellular component of the total influx can be easily distinguished from the cellular fraction by inhibiting the latter with SCN^- added during the measuring time (45 sec). The paracellular component was measured in this way under all the conditions tested, generally without significant changes. Amiloride addition was the only exception as a small but significant increase of the SCN^--insensitive influx was observed. An amiloride-induced decrease in cell volume is unlikely to explain the reduction of paracellular Cl^- influx, since in other experiments (e.g., incubation with HCO_3^--free saline), a similar reduction of cell volume is likely, but there was no effect on SCN^--insensitive Cl^- influx. A probable explanation for the amiloride effect is titration of the negative charges of the paracellular route (Balaban, Mandel & Benos, 1979), which might reduce the hindrance for Cl^- and increase the paracellular influx.

The cellular component of Cl^- influx was calculated by subtracting the SCN^--insensitive fraction to the total influx measured. The experiments here reported confirm that it is entirely due to a neutral coupling with Na^+, but that this coupling exhibits a double nature.

This is in agreement with what was previously found from the analysis of the complex kinetics of Cl^- entry (Cremaschi et al., 1983). The body of evidence in favor of this thesis is the following:

a) The removal of bicarbonate from the bathing salines reduces cell Cl^- influx only to about 50%.

[1] Either Na^+ absence or SCN^- presence reduces the radiochemical exchangeable pool of intracellular Cl^- to values approximating to zero; similarly, lumen to epithelium Cl^- influx is reduced to minimal values that are not significantly different from each other.

Since bicarbonate was absent from both bathing salines for 45 min and salines were bubbled in this time with 100% O_2, CO_2 produced by the cells was rapidly removed and the epithelium was deprived from endogenous bicarbonate as well. The fact that 10^{-4} M acetazolamide also inhibits only about 50% of cell Cl^- influx in the presence of bicarbonate confirms that the bicarbonate-sensitive fraction of cell Cl^- influx is only about 50% and that the tissue is effectively depleted from endogenous bicarbonate in the bicarbonate removal experiments.

b) Furosemide reduces cell Cl^- influx only to about 50% when it is added on the luminal side even at high doses and for a long time. Its maximal action is already obtained with a 10^{-5} to 10^{-4} M concentration and with exposures for a few seconds. In other tissues, at these concentrations furosemide inhibits Cl^-/HCO_3^- exchange. Only at higher concentrations it inhibits Na^+-K^+-$2Cl^-$ cotransport (Aronson & Seifter, 1984) and carbonic anhydrase (Vogh & Langham, 1981). These observations and the fact that furosemide in rabbit gallbladder is ineffective in the absence of HCO_3^- indicate that Cl^- entry into the cell is related to a Cl^-/HCO_3^- exchange, but that this process accounts for only about half of Cl^- influx.

c) SITS, which is an inhibitor of Cl^-/HCO_3^- exchange (Knauf, 1979), again reduces cell Cl^- influx only to about 50% even with high doses and for a long time of exposure. Moreover, its action is not additive to that of furosemide and is exerted (as expected) on the bicarbonate-sensitive fraction of Cl^- influx.

d) Amiloride also inhibits only about 50% of cell Cl^- influx. It could be that maximal effects were not reached with the dose used (10^{-3} M) due to the high concentration of competing Na^+ present in the bathing saline. However, the action of amiloride was not potentiated by maximal doses of SITS. This should be sufficient evidence that a Na^+/H^+ exchange has been inhibited (to a maximal or nonmaximal extent) with the consequence of an indirect maximal inhibition of a Cl^-/HCO_3^- antiport which again seems to account for only about 50% of Cl^- entry.[2]

e) Luminal Na^+ removal during the measuring time (45 sec) has different effects on Cl^- uptake depending on the presence of bicarbonate. If bicarbonate is absent, Na^+ removal immediately abolishes Cl^- influx: this is consistent with a coupling of Cl^- and Na^+ on a single carrier. In contrast, in the presence of bicarbonate cellular Cl^- influx is eliminated by Na^+ removal in a time-dependent way. Thus, under this condition cellular Cl^- influx could be entirely due to a Cl^-/HCO_3^- exchange functionally related to a Na^+/H^+ antiport or, alternatively, could be due to coexistence of Na^+-Cl^- symport and double exchange.

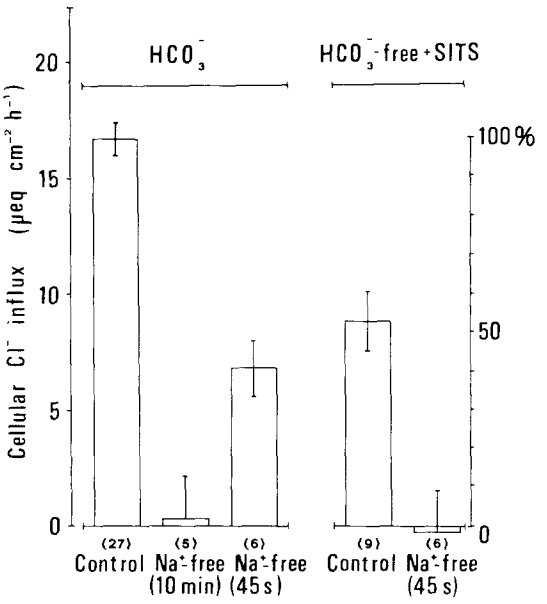

Fig. 8. Effects of luminal Na^+ removal. Experiments in which HCO_3^- was present in the bathing salines (45 min) are shown on the left of the Figure; those in which HCO_3^- was absent (45 min) and 10^{-4} M SITS was added during the measuring time (45 sec) are on the right. The paracellular component, measured as SCN^--insensitive fraction under the corresponding experimental condition, has already been subtracted and does not appear in the Figure

Together these observations indicate that Na^+-Cl^- transport across the apical membrane of the epithelium of rabbit gallbladder is due to a double exchange of Na^+/H^+ and Cl^-/HCO_3^- and to a symport on a single carrier each accounting for about 50% of the transport.

Comparisons with the Results Obtained in Other Animal Species

The existence of a double exchange in guinea pig and *Necturus* gallbladder is well documented (Ericson & Spring, 1982; Heintze & Petersen, 1980; Weinman & Reuss, 1982, 1984; Reuss & Costantin, 1984). Heintze et al. (1981) proposed hypothetically that the double exchange might be the only

[2] If Na^+/H^+ exchange in rabbit gallbladder has the same K_m for Na^+ and K_i for amiloride of the corresponding transport present in rabbit renal microvillus membane (Kinsella & Aronson, 1981), one can calculate that only 60% of the exchange has been inhibited by 10^{-3} M amiloride at the 143 mM Na^+ concentration of the bathing saline. However, the rate of Na^+/H^+ exchange in rabbit gallbladder is greater than that of Cl^-/HCO_3^- exchange. In fact, the former is responsible for the transepithelial transport of $NaHCO_3$ as well as of that fraction of NaCl which is related to the Cl^-/HCO_3^- exchange (Cremaschi et al., 1979). Thus, a complete inhibition of the latter antiporter does not require a full inhibition of the former.

mechanism involved in neutral Na^+-Cl^--coupled entry even when bicarbonate is absent from the bathing salines; in this case it should be supported by bicarbonate endogenously produced. Further studies on this topic were undertaken in *Necturus* gallbladder. Machen and Zeuthen (1983) and Reuss (1984) produced evidence that the double exchange is "the predominant or the sole mechanism of apical membrane NaCl entry" in that epithelium. Moreover, the double exchange measured by these authors accounts for about the entire transepithelial transport. Finally, Reuss did not observe any effect of mucosal K^+ removal or bumetanide on NaCl entry into the cell. This result would rule out the presence of a Na^+-K^+-$2Cl^-$ cotransport.

In complete disagreement with these data, Ericson and Spring (1982) and Spring (1984) proposed that the double exchange is activated only when cell volume regulation begins due to the presence of a hypertonic medium, whereas under normal conditions the transepithelial transport is supported by a NaCl entry due only to a Na^+-Cl^- cotransport on a single carrier. This process would be inhibited by bumetanide and insensitive to K^+.

These different findings on *Necturus* gallbladder are difficult to reconcile. Our results on rabbit gallbladder are qualitatively in agreement with the experiments of Machen and Zeuthen (1983) and Reuss (1984), since we observe consistently double exchange under isotonic conditions. A possible objection is that all our manipulations would produce cell shrinkage which would activate the double exchange. Although this could be the case when we remove luminal Na^+ for 10 min, we can observe that: a) treatment with SITS and furosemide at low doses in the presence of bicarbonate produces an inhibition of Cl^- influx which can only be the cause and not the result of a shrinkage; b) HCO_3^- removal certainly causes cell shrinkage (Cremaschi et al., 1979), but under these conditions Cl^- influx is reduced (not increased) and the residual Cl^- influx turns out to be insensitive to the typical inhibitors of Cl^-/HCO_3^- antiport (SITS, furosemide).

The apical double exchange we observed not only seems to be present under steady-state conditions and unrelated to transient volume regulations, but also appears to be involved in transepithelial transport as observed by Reuss (1984) and Machen and Zeuthen (1983) for *Necturus*. It is noteworthy that HCO_3^- removal reduces to 57% NaCl transepithelial transfer (Cremaschi et al.; 1979) and a quantitatively similar effect is produced by inhibitors of carbonic anhydrase (Wheeler, Ross & King, 1969). These effects are quantitatively comparable to the corresponding effects here reported on cellular Cl^- influx so that the abolishment of Cl^-/HCO_3^- exchange, which accounts for about 50% of Cl^- entry through the apical membrane, seems to reduce to about 50% Cl^- transepithelial transport.

On the other hand, although our results indicate that double exchange is related to transepithelial transport, in rabbit gallbladder this process does not appear to be the only mechanism for NaCl entry. Cellular Cl^- influx, measured in the absence of exogenous bicarbonate, is not affected by SITS, furosemide or acetazolamide so that it does not appear to be due to a residual double exchange related to endogenous bicarbonate. The Cl^- influx cannot be the result of a Cl^- conductance as such a conductance is absent from this membrane both when HCO_3^- is and is not present in the bathing salines (*see* Introduction). Finally the Cl^- influx is immediately sensitive to Na^+ removal. In conclusion, about 50% of Cl^- entry seems to be due to a real Na^+-Cl^- symport on a single carrier. The reason for the different quantitative importance of the double exchange between *Necturus* and rabbit gallbladder may be related to the large difference in transepithelial transport rates between the gallbladders of these two animal species. NaCl transepithelial transport is equal to 1.35 μeq cm^{-2} hr^{-1} in *Necturus* gallbladder at room temperature (Reuss, 1984) and to 12.2 μeq cm^{-2} hr^{-1} in rabbit gallbladder at 27°C (Cremaschi et al., 1979) in the presence of HCO_3^-. When this anion is removed, the transepithelial transport decreases by about 36% (Reuss, 1984), that is to 0.86 μeq cm^{-2} hr^{-1} in *Necturus*; this value is quite small so that the net entry into the cell might be due to a double exchange even under these conditions, with the support of endogenous bicarbonate. The corresponding transport in rabbit gallbladder is equal to 7 μeq cm^{-2} hr^{-1} (Cremaschi et al., 1979), a value always quite high which hardly could be supported by endogenous bicarbonate. To emphasize the high rate of NaCl transport in rabbit gallbladder, it is interesting to note that in rabbit ileum Na^+ transport is equal to 2.8 μeq cm^{-2} hr^{-1} at 38°C and when HCO_3^- is present (Schultz & Zalusky, 1964).

Further Observations

Two major points need further discussion. First, the Na^+-Cl^- symport on a single carrier might be present simultaneously with the double exchange when HCO_3^- is in the bathing salines: in this case the total Cl^- entry into the cell has two components. Alternatively, Na^+-Cl^- symport could turn up as a homeostatic reaction of the cell in response to all the experimental conditions abolishing the double exchange: in this case the total Cl^- entry into the cell has only one component in each occasion. The

experiments reported above do not permit unequivocal discrimination between the two hypotheses. However, the high rate with which the homeostatic reaction would occur under many of our experimental conditions (e.g. SITS or furosemide treatment) suggests the first hypothesis to be more sound. The use of isolated apical membrane vesicles might shed some light on this problem.

The second observation pertains to the nature of the Na^+-Cl^- symport on a single carrier. It could be in fact a Na^+-K^+-$2Cl^-$ cotransport, as it is in the majority of the cells in which the symport is found. This point is the object of the experiments report in the next paper. However, among the results here reported, one can offer a preliminary suggestion on this topic. Since furosemide at high doses is a typical inhibitor of Na^+-K^+-$2Cl^-$ cotransport (Aronson & Seifter, 1984) and the experiments here reported show that it is unable to inhibit the HCO_3^--insensitive fraction of Cl^- influx even at 10^{-3} M, the observed Na^+-Cl^- symport is likely to be independent of K^+.

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