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Nature of the Neutral Na^+-Cl^- Coupled Entry at the Apical Membrane of Rabbit Gallbladder Epithelium: IV. Na^+/H^+, Cl^-/HCO_3^- Double Exchange, Hydrochlorothiazide-Sensitive Na^+-Cl^- Symport and Na^+-K^+-$2Cl^-$ Cotransport are all Involved

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Summary. Transepithelial fluid transport was measured gravimetrically in rabbit gallbladder (and net Na^+ transport was calculated from it), at 27°C, in HCO_3^--free bathing media containing 10^{-4} M acetazolamide. Whereas luminal 10^{-4} M bumetanide or 10^{-4} M 4-acetamido-4′-iso-thiocyanostilbene-2,2′-disulfonate (SITS) did not affect fluid absorption, 25 mM SCN^- abolished it; hydrochlorothiazide (HCTZ) in the luminal medium reduced fluid absorption from 28.3 ± 1.6 (n = 21) to 8.6 ± 1.6 $\mu l\ cm^{-2}\ hr^{-1}$ (n = 10), i.e., to about 30%. This maximum effect was already obtained at 10^{-3} M concentration; the apparent IC_{50} was about 2×10^{-4} M. The residual fluid absorption, again insensitive to SITS, was completely inhibited by SCN^- or bumetanide. Cl^- influx at the luminal border of the epithelium, measured under the same conditions and corrected for the extracellular space and paracellular influx, proved insensitive to 10^{-4} M bumetanide, but was slowly inhibited by 10^{-3} M HCTZ, with maximum inhibition (about 54%) reached after a 10-min treatment; it subsequently rose again, in spite of the presence of HCTZ. However, if the epithelium, treated with HCTZ, was exposed to 10^{-4} M bumetanide during the measuring time (45 sec), inhibition was completed and the subsequent rise of Cl^- influx eliminated. Intracellular Cl^- accumulation with respect to the predicted activity value at equilibrium decreased significantly upon exposure to 10^{-3} M HCTZ, reached a minimum within 15–30 min of treatment, then rose again significantly at 60 min. Simultaneous exposure to HCTZ and bumetanide decreased the accumulation to a significantly larger extent as compared to HCTZ alone, already in 15 min, and impeded the subsequent rise. Intracellular K^+ activity rose significantly within 30 min treatment with HCTZ; the increase proved bumetanide dependent.

The results obtained show that Na^+-Cl^- symport, previously detected under control conditions, is the HCTZ-sensitive type; its inhibition elicits bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransport. Thus, the three forms of neutral Na^+-Cl^--coupled transport so far evidenced in epithelia, Na^+/H^+, Cl^-/HCO_3^- double exchange (in the presence of exogenous bicarbonate), HCTZ-sensitive Na^+-Cl^- symport and bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransport, are all present in the apical membrane of rabbit gallbladder.

Key Words bumetanide · SITS · SCN^- · intracellular ion activities · fluid transport · Cl^--influx · homeostatic regulation

Introduction

Using different techniques, we have accumulated evidence that about 50% of the neutral Na^+-Cl^- coupled entry at the apical membrane of rabbit gallbladder epithelium is independent of the presence of HCO_3^- and K^+, insensitive to stilbenes, amiloride, acetazolamide, furosemide and bumetanide, sensitive to SCN^- and immediately abolished by the removal of Na^+ (Cremaschi et al., 1983; 1987*a,b*). On this ground, we have concluded that this fraction should correspond to a Na^+-Cl^- symport on a single carrier, identical to that originally hypothesized when neutral Na^+-Cl^- entry was found in epithelia (Nellans, Frizzell & Schultz, 1973; Cremaschi, Hénin & Ferroni, 1974; Cremaschi & Hénin, 1975; Frizzell, Dugas & Schultz, 1975; Hénin & Cremaschi, 1975).

In recent years, two Na^+-Cl^- symports on a single carrier, independent of K^+, have been discovered. One is associated with Na^+-K^+-$2Cl^-$ cotransport, represents a modulation of this system and is sensitive to furosemide and bumetanide (Eveloff & Warnock, 1987); the second has been found in fish urinary bladder (Duffey & Frizzell, 1984; Stokes, 1984) and mammal distal tubule (Costanzo & Windhager, 1978; Velasquez, Good & Wright, 1984), and is sensitive to hydrochlorothiazide (HCTZ). The insensitivity to furosemide and bumetanide strongly suggests that the symport present in gallbladder is

similar to that of urinary bladder and distal tubule; it therefore also seemed important to ascertain the effects of HCTZ on this epithelium.

We have also confirmed the existence of Na^+-Cl^- symport in rabbit gallbladder with isolated apical membrane vesicles (Bottà et al., 1987; Meyer et al., 1990); in this case, the preliminary results indicated that the symport was inhibited by HCTZ (Meyer et al., 1990).

The aim of the present paper is to report a detailed investigation into the effects of HCTZ on transepithelial and apical transport of ions "in vitro" on the intact epithelium of rabbit gallbladder.

All the experiments were performed in the absence of bicarbonate in the bathing salines so as to eliminate the Na^+/H^+, Cl^-/HCO_3^- double exchange component of the neutral NaCl entry (Cremaschi et al., 1987*a*,*b*) and to investigate Na^+-Cl^- symport only. Care was also taken to avoid endogenous bicarbonate production, when necessary, as will be described.

The results obtained were presented in preliminary form at the Autumn Meeting of the Italian Physiological Society (Cremaschi et al., 1990).

Materials and Methods

Male New Zealand rabbits (body weight 3–3.5 kg) were purchased from Azienda Agricola Bernasconi (Valmorea, Como, Italy). They were killed by cervical dislocation, and the gallbladders were excised and washed free of bile with Krebs-Henseleit solution.

Fluid Absorption Measurement

The gallbladder (noneverted preparation) was cannulated, filled with Krebs-Henseleit solution, bathed with the same solution on the serosal side and then incubated for half an hour to allow the tissue to recover from the isolation shock. Then it was emptied, washed nine times, filled with the test solution and weighed. Fluid absorption was measured gravimetrically every 30 min for two experiment periods and expressed as $\mu l\ cm^{-2}\ hr^{-1}$. Only the results of period 2 were taken into account; period 1 variation in weight was ignored, since it was possibly affected by changes in cell volume due to the change in saline. In many further sets of experiments fluid absorption was measured for four experiment periods to observe the time-course of transfers and inhibitions. In this case the results of all four experiment periods were reported in Fig. 4; of course, the value of period 1 may be affected by the same error, as mentioned above.

Na^+ absorption was calculated from the measured net fluid flow, the latter being known to be a transfer of an isotonic saline of Na^+ salts.

Measurement of Cl^- Uptake

The method used was very similar to that previously reported (Cremaschi et al., 1983, 1987*a*). Briefly, gallbladders, opened flat, were mounted, carefully avoiding any stretch, on a nylon mesh between two Lucite chambers, with the luminal surface facing upwards (exposed area: 0.6 cm^2). The luminal solution (0.8 ml) was agitated 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 on both sides for 45 min in bicarbonate-free saline. Then it was washed five 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. The variations in incubation on the luminal side will be described later. At the end of the experiment the luminal medium was withdrawn, the tissue squeezed with an ice-cold isotonic solution of mannitol and punched off. It was then bathed in 2 ml of bidistilled water, frozen at −20°C, thawed and boiled for 15 min. After centrifuging the material was weighed to calculate the exact total water. Samples in triplicate of the supernatant and of the incubation fluid were assayed for radioactivity by a liquid scintillation spectrometer (Minaxi β-Tri-Carb, 4000 series Packard Instruments, Inc., Zurich). The measured influx was expressed as $\mu mol\ cm^{-2}\ hr^{-1}$.

Measurement of Intracellular K^+ and Cl^- Activities

K^+ or Cl^- sensitive theta-microelectrodes (one conventional and one ion-selective channel) with tips of 0.2 to 0.3 μm were prepared by the method previously described (Meyer et al., 1985).

The K^+- and Cl^--sensitive resins (labeled 477317 and 477913, respectively) came from Corning (Corning NY) Pyrex theta tubing was purchased from R.D. Scientific Glass (Spencerville, MD) K^+- and Cl^--sensitive microelectrode channels were backfilled with 154 mM KCl; the conventional channels were backfilled with 500 mM KCl. Each electrode was calibrated in isosmotic test solutions of NaCl-KCl, for K^+-sensitive resins, and NaCl-sodium gluconate, for Cl^--sensitive resins; the sums of the Na^+ and K^+ or Cl^- and gluconate activities were kept constant at 112.4 mM (total concentration = 154 mM). When the K^+ or Cl^- activity of the calibration solution was changed from 112.4 to 11.2 mM, the voltage of the selective channel changed by about 52 mV in both cases and that of the conventional channel (tip potential and diffusion potential together) was less than 1 mV. The resistances of the K^+- and Cl^--sensitive channels were 3–8 × 10^9 and 5–10 × 10^9 Ω, respectively. The Cl^- resin did not demonstrate dependence on phosphate and sulfate when these anions were present at the concentration of the incubation solutions used, or at what is presumably the cell concentration. Moreover, the Cl^- resin was insensitive to 5 × 10^{-5} M bumetanide and, for about 20 min continuous exposures, to 10^{-3} M HCTZ. K^+ resin was insensitive to all the drugs used.

The set-up for potential recordings was similar to that previously reported (Cremaschi et al., 1984, 1987*b*). Briefly, the two shanks of the microelectrode were connected through a dual-channel high impedance electrometer (model FD223, World Precision Instruments, New Haven, CT) to a two-line strip chart recorder (PM8262 Xt Recorder, Philips, Eindhoven, The Netherlands). Short pulses of direct current (20 μA), generated by a Junior Stimulator (model 8049, C.F. Palmer, London, UK) were passed through the tissue every 10 sec by means of Ag/AgCl electrodes in order to check microelectrode entrance into the cell from the voltage deflections. The mounted tissue separated an upper mucosal from a lower serosal Lucite chamber (window: 0.17 cm^2), both perfused (12 ml/min) by gravity and kept inclined so as to minimize fluctuations of the fluid level on the epithelium;

the fluid was finally collected in a beaker and sent back to the reservoir by a peristaltic pump (Minipuls 2, Gilson, Villiers le Bel, France) so as to keep the reservoir level constant. The time for the renewal of the fluid on the epithelium was 4 sec.

Intracellular activities were measured with sets of multiple short impalements (each about 20 sec, after stability was reached), performed at different experiment times. The microelectrode was withdrawn from the saline containing HCTZ during the intervals between the different sets of impalements and immersed in a drug-free saline; its response was again checked at the end with calibration solutions. Prolonged single impalements were prevented by the experimental observations protracted for 90 min and by the interference of HCTZ with the Cl^- resin upon long exposures together with the amphipathic properties of this drug which perhaps might enter the cell. General criteria for impalement validation were: (i) the potential differences (PDs) measured by both electrode shanks should change abruptly as well as the voltage deflections, (caused by the d.c. pulses) which should increase, (ii) after the stability of the PDs was reached, signals had to remain constant for about 20 sec; finally the PDs should return rapidly to the respective baselines on leaving the cell. Voltage readings during impalements were stable generally within 6–7 sec with the conventional or the K^+-selective channel, within 10–11 sec with the Cl^- channel (Figs. 7 and 8); in about 20% of the initially successful impalements stabilization was not reached within 10 sec for K^+-selective shanks, so impalements were stopped and discarded. Conversely, a stabilization time of even 15 sec was accepted for Cl^--sensitive shanks. During calibrations signals from K^+- or Cl^--selective shanks were stable within 3–4 and 6–7 sec, respectively.

Salines

The Krebs-Henseleit saline, used only for preliminary washings and the 30 min preincubation, had the following composition (in mM): 142.9 Na^+, 5.9 K^+, 2.5 Ca^{2+}, 1.2 Mg^{2+}, 127.7 Cl^-, 24.9 HCO_3^-, 1.2 SO_4^{2-}, 1.2 $H_2PO_4^-$; pH 7.4. The bicarbonate-free saline (phosphate saline) contained (in mM): 145.3 Na^+, 6.2 K^+, 2.5 Ca^{2+}, 1.2 Mg^{2+}, 125.3 Cl^-, 13.7 SO_4^{2-}, 12.5 mannitol, 2.8 HPO_4^{2-}, 0.6 $H_2PO_4^-$; pH 7.4. Inhibitors were added directly to the solution (since the change in osmolality was negligible), with the exception of SCN^- (25 mM) which was substituted for 12.5 mM SO_4^{2-} and mannitol. HCTZ was directly dissolved in the saline and the final concentration was checked with a spectrophotometer (Lambda 5, Perkin Elmer, Norwalk, CT) at the wavelength of 226 nm. The salines were bubbled with 95% O_2 + 5% CO_2 (Krebs-Henseleit saline) or 100% O_2 (phosphate saline). The incubation temperature for all the experiments was 27 ± 1°C.

Materials

SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate), HCTZ and acetazolamide were purchased from Sigma (St. Louis, MO); SCN^- from Carlo Erba-Farmitalia (Milan, Italy); bumetanide was kindly donated by Leo Pharmaceutical Products (DK-2750 Ballerups, Denmark); ouabain was supplied by E. Merck (Darmstadt, FRG). All other reagents used were of AR grade.

Statistics

The results are presented as means ± SEM with the number of experiments in parentheses. Statistical probability was analyzed with Student's *t*-test; when possible, paired data analysis was used.

Results

Fluid and Na^+ Absorption

When the test solution was an acetazolamide-free phosphate saline, the rate of fluid loss from the gallbladder was 39.9 ± 2.3 $\mu l\ cm^{-2}\ hr^{-1}$ (6 expt.); with 10^{-4} M acetazolamide in the mucosal medium, it was 34.1 ± 1.6 $\mu l\ cm^{-2}\ hr^{-1}$ (21 expt.). Though these values are not significantly different, the statistical probability is very close to 0.05; this suggests that bicarbonate traces were probably present and the Na^+/H^+, Cl^-/HCO_3^- double exchange was likely to be slightly activated when acetazolamide was not added to the saline. Since the gallbladder sac preparation was necessarily bubbled with 100% O_2 only on the serosal side, the endogenous CO_2, diffused into the unstirred and nonoxygenated luminal medium, might have been removed too slowly and generated bicarbonate. On this ground, in the subsequent experiments the test solution was always basically a bicarbonate-free saline, to which 10^{-4} M acetazolamide was added on the mucosal side.

Every set of experiments was followed by an equivalent set with ouabain (5×10^{-4} M) present in the serosal medium. Maximum inhibition of the rate of fluid loss was reached by the second experiment period. The small residual rate of fluid loss was considered a passive phenomenon and subtracted from the corresponding loss rate, measured in the absence of ouabain.[1] The several sets of passive, ouabain-independent losses obtained were not significantly different from each other or differed by only a few microliters; pooled together, they gave a mean of 8.1 ± 0.7 $\mu l\ cm^{-2}\ hr^{-1}$ (39 expt.). All the results

[1] This procedure implies that 5×10^{-4} M ouabain produces a complete inhibition of active electrolyte transport. However, Sullivan and Berndt (1973) have shown that ouabain only reduces luminal acidification to 1/3 of the control in a phosphate-buffered saline similar to the one used here. On the other hand, luminal acidification is entirely suppressed by Na^+ removal (Cremaschi et al., 1979). All this may suggest that the small residual fluid transport is not passive. Yet, it is to emphasize that H^+ secretion in phosphate-buffered saline is 0.23 $\mu mol\ cm^{-2}\ hr^{-1}$, equivalent to 0.5 $\mu l\ cm^{-2}\ hr^{-1}$ fluid transport against the mean residual fluid loss here reported of 8.1 $\mu l\ cm^{-2}\ hr^{-1}$. Thus, the possible residual active fluid transport is definitely negligible compared to the residual, ouabain-independent loss of fluid.

Moreover, Van Os and Slegers (1971) have found an inhibition of the cellular ATPase in the epithelium of rabbit and guinea-pig gallbladders which was not significantly different by either removing Na^+ or treating with ouabain at a concentration five times less the one used here; this indicates that the Na^+/K^+-ATPase was actually already completely inhibited by ouabain at a 10^{-4} M concentration.

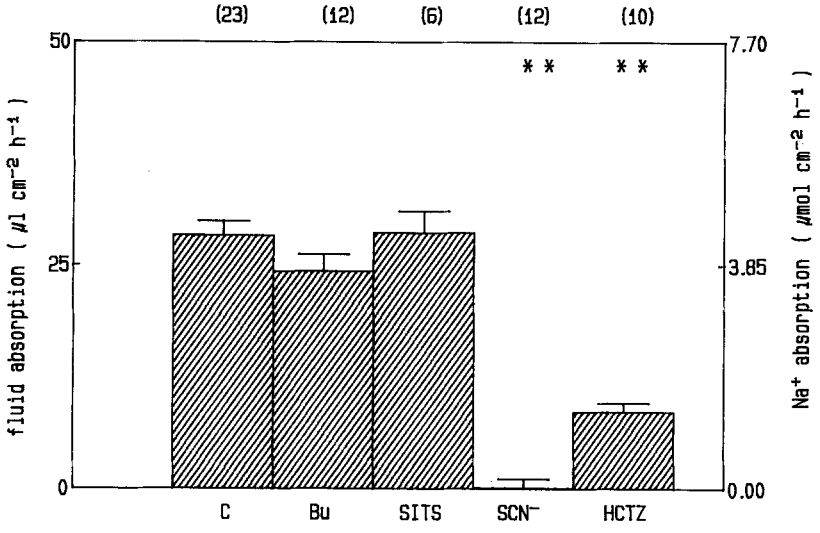

Fig. 1. Fluid and Na^+ absorption measured in bicarbonate-free bathing media, containing 10^{-4} M acetazolamide. Effects of different inhibitors added to the luminal medium are shown. *C* = control; *Bu* = bumetanide, 10^{-4} M; SITS, 10^{-4} M; SCN^-, 2.5×10^{-2} M; HCTZ, 1.8×10^{-3} M. Number of experiments is shown in parentheses. $^{**}P < 0.01$ relative to control.

reported below represent only the secondarily active transport dependent on ouabain.

Bumetanide (10^{-4} M) or SITS (10^{-4} M), added to the mucosal medium, did not significantly influence Na^+ and fluid absorption (Fig. 1). This was to be expected on the basis of the results previously obtained by measuring Cl^- influx through the apical membrane and intracellular Na^+ and Cl^- activities under the same conditions, those parameters not being affected by these drugs (Cremaschi et al., 1987 *a,b*). Conversely, thiocyanate (25 mM), present in the lumen, completely abolished Na^+ and fluid absorption (Fig. 1), again in agreement with the findings previously reported (Cremaschi, Hénin & Meyer, 1979).

Having ascertained that the preparation response was consistent with the results previously obtained, HCTZ was tested at a 1.8×10^{-3} M luminal concentration, a rather high value on the basis of the literature. Figure 1 shows that under these conditions fluid absorption is significantly inhibited by 69.6%. A statistically similar decrease to 8.9 ± 2.7 $\mu l\ cm^{-2}\ hr^{-1}$ (4 expt.) was obtained with 1.8×10^{-3} M HCTZ present on both sides.

The diuretic, added only to the luminal medium, was then tested at different concentrations. The outcome is shown in Fig. 2. At 10^{-4} M concentration the inhibition was highly significant ($P < 0.01$), and equal to 19.7%. The above-reported inhibition of 69.6% with 1.8×10^{-3} M HCTZ was the maximum obtained; thus about 30% of the transport proved insensitive to HCTZ. The dose-response relationship, expressed as a logarithm of the dose against inhibition percentage, did not readily fit a sigmoidal curve, thereby suggesting interference by complex factors with the inhibition. The data points reported in Fig. 2 were then interpolated with a hand-drawn curve, graphically giving a dose apparently able to produce 50% of the maximum inhibition (IC_{50}) of about 2×10^{-4} M. The apparent maximum inhibition was achieved at about 10^{-3} M concentration.

Further experiments were carried out to elucidate the nature of the residual HCTZ-insensitive Na^+ and fluid absorption. All the experiments were conducted in the presence of 1.8×10^{-3} M HCTZ. The results are reported in Fig. 3. Treatment with 5×10^{-4} M SITS added to the luminal medium was ineffective, as expected. Again, as was likely, 25 mM SCN^- in the lumen significantly inhibited the transport to a value not significantly different from zero. Unexpectedly, however, 10^{-4} M bumetanide on the luminal side also completely abolished the transfer of fluid and Na^+ in spite of the fact that, in the absence of HCTZ, it was ineffective (Fig. 1).

In the final groups of experiments the measurements continued for four experiment periods so that the time course of the transport and possible inhibitions could be observed in detail. Figure 4 shows the results obtained. Under control conditions fluid absorption slowly and significantly decreased in two hours, from 30.2 to 20.3 $\mu l\ cm^{-2}\ hr^{-1}$. When 10^{-4} M bumetanide was present in the lumen during the two observation hours, the values obtained were not significantly different from the controls at any time. Conversely, if 1.8×10^{-3} M HCTZ was dissolved in the luminal medium during the two observation hours, fluid transport was already inhibited ($P < 0.01$) in the first experiment period from 30.2 (the control value quoted above) to 12.6 $\mu l\ cm^{-2}\ hr^{-1}$; then it further decreased to 8.6 $\mu l\ cm^{-2}\ hr^{-1}$ in the second experiment period, and subsequently increased, progressively and significantly, to reach a value of 13.9 $\mu l\ cm^{-2}\ hr^{-1}$ in the final period which was not significantly different from the correspond-

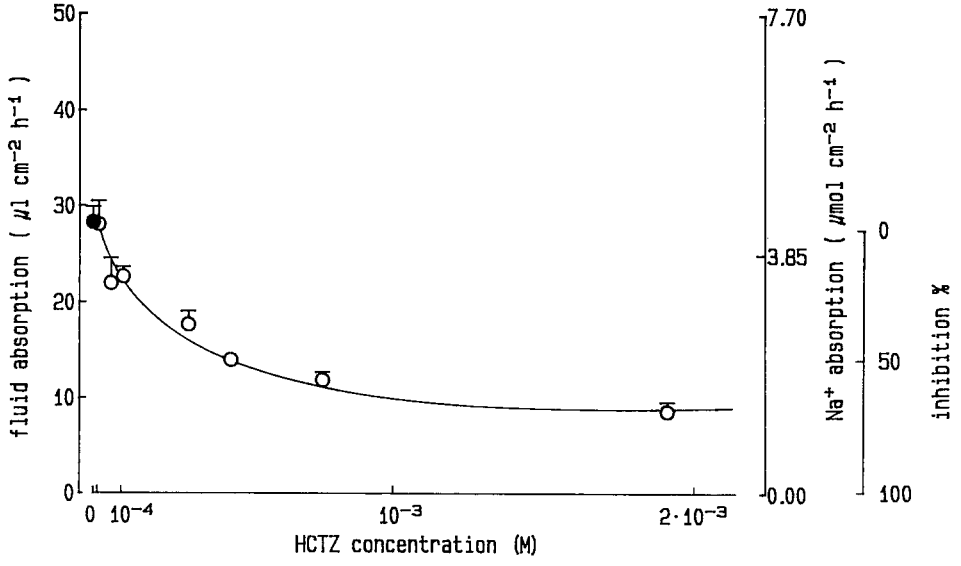

Fig. 2. Fluid and Na^+ absorption: effects of HCTZ added to the luminal medium at different concentrations. Experimental conditions were the same as in Fig. 1. Filled circle: control (n = 23). Open circles: treated tissues (n = 4–15 for each data point).

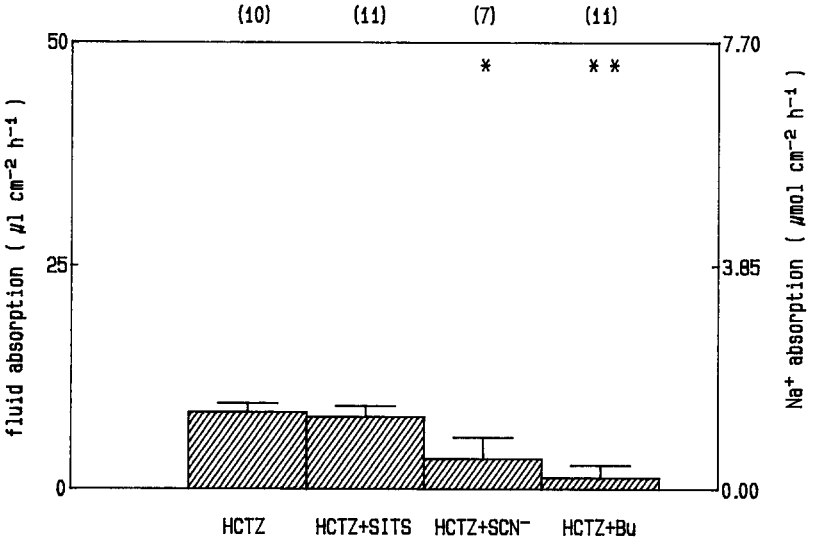

Fig. 3. Residual HCTZ-insensitive fluid and Na^+ absorption: effects of different inhibitors. HCTZ, 1.8×10^{-3} M; SITS, 5×10^{-4} M; SCN^-, 2.5×10^{-2} M; *Bu* = bumetanide, 10^{-4} M. Experimental conditions were the same as in Fig. 1. Number of experiments is shown in parentheses. * and ** represent $P < 0.05$ and $P < 0.01$ relative to HCTZ condition.

ing control. These results show that the initial inhibition of the absorption by HCTZ was spontaneously eliminated with time. However, if both 1.8×10^{-3} M HCTZ and 10^{-4} M bumetanide were simultaneously present in the lumen, Na^+ and fluid transports were already completely abolished in the second period and remained so throughout the experiment. Altogether, these results show that HCTZ completely inhibits the original, bumetanide-insensitive absorption and the inhibition in some way elicits a new bumetanide-sensitive transport, inactive under control conditions (indicated by the hatched area in Fig. 4). At the end, the effect of HCTZ is even entirely masked.

Cl^- Influx through the Apical Membrane

Cl^- influx at the apical membrane was also measured, to give a more direct determination of the effects of HCTZ on Na^+-Cl^- cotransport. The cell fraction on the total influx was identified by treating the luminal side of the tissue with 25 mM SCN^- during the 45-sec measuring time, it being known

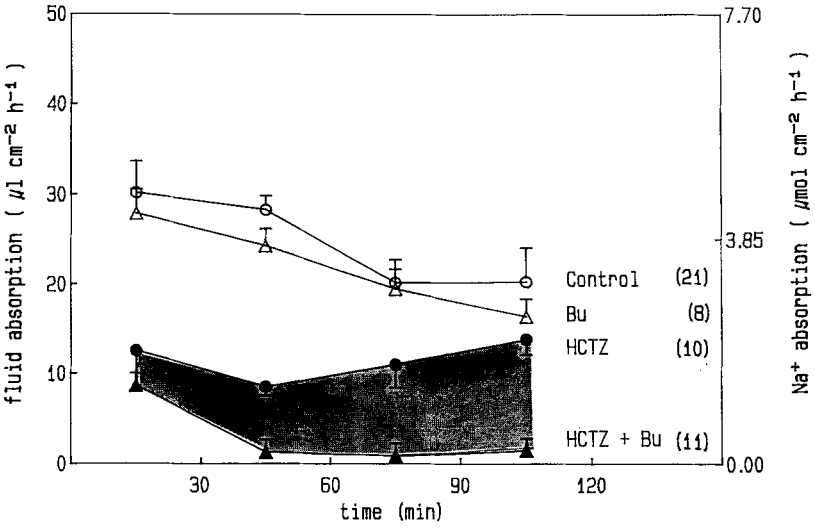

Fig. 4. Time course of the effects of luminal bumetanide (*Bu*, 10^{-4} M) and HCTZ (1.8×10^{-3} M) on fluid and Na^+ absorption. The two drugs were present for all the four experimental periods. The hatched area indicates the bumetanide-sensitive tarnsport, residual upon HCTZ treatment. Number of experiments is shown in parentheses.

that the entire cellular Cl^- entry is neutrally coupled with Na^+ and completely inhibitable with SCN^- (Cremaschi & Hénin, 1975; Cremaschi et al., 1979, 1983, 1987*a*,*b*). Thus, for each set of experiments, a second set with SCN^- in the luminal saline was carried out to directly determine the paracellular component of the influx (SCN^--insensitive fraction) to be subtracted from the measured total influx. The SCN^--insensitive fraction was not significantly different in the various situations tested, although in the gallbladders treated with HCTZ or HCTZ and bumetanide this fraction tended to increase by 1–2 μmol cm^{-2} hr^{-1}; the measurements obtained, pooled together, gave an influx of 5.8 ± 0.5 μmol cm^{-2} hr^{-1} (45 expt.), a value not significantly different from that previously reported (Cremaschi et al., 1987*a*). All the values reported below refer directly to the SCN^--sensitive, cellular influx.

In none of these experiments was acetazolamide added to the luminal medium, since it had already been demonstrated that under these experiment conditions it does not significantly affect Cl^- influx (Cremaschi et al., 1987*a*). Probably the gallbladder sheet preparation used in this case, with both sides gassed with 100% O_2, ensures sufficient removal of the endogenous CO_2, so as to prevent significant bicarbonate formation.

In the first set of experiments the influence of 10^{-4} M bumetanide, present in the luminal medium during the measuring time (45 sec), was checked. Figure 5 shows that bumetanide was ineffective on Cl^- influx, as was expected (Cremaschi et al., 1987*b*).

In the second set of experiments 10^{-3} M HCTZ was added to the luminal saline during the measuring time (45 sec) and during a preincubation period of 45 min so as to reproduce a prolonged treatment situation like that used during fluid transport measurements. Figure 5 shows that Cl^- influx was significantly inhibited by 43.1%. However, when the tissue was treated in the same way and for the same time with HCTZ, but 10^{-4} M bumetanide was also present in the lumen during the measuring time (45 sec), Cl^- influx decreased to a value not statistically different from zero.

The time course of the effect of HCTZ on Cl^- influx was then investigated. To this end, the influx was measured in the presence (luminal side) of 10^{-3} M HCTZ during the measuring time (45 sec) and during preincubation periods, varying in length from 0 to 45 min. The influx was always determined at the same experiment time (i.e., after 45 min incubation in phosphate saline). The results are shown in Fig. 6. Unexpectedly, HCTZ, added to the medium only during the measuring time (45 sec), had no effect; it was only at 5 min that the inhibition became significant ($P < 0.05$). Maximum inhibition was observed at 10 min (about 54%, $P < 0.01$). Then, Cl^- influx rapidly rose, again reaching the control value at 15 min; it subsequently decreased again, to reach the value already reported above, at a 45-min preincubation time. When the experiment was repeated, the tissue being treated with HCTZ in the same way and for the same preincubation periods, but 10^{-4} M bumetanide being added to the luminal bath during the measuring time (45 sec), the inhibition of Cl^- influx was statistically equal to that obtained with HCTZ alone for 10 min; with longer preincubation times with HCTZ, Cl^- influx was abolished and remained so for 45 min.

From these results it is evident that: (i) HCTZ is not able to inhibit Cl^- influx immediately; (ii) a bumetanide-sensitive Cl^- influx, absent under control conditions, is obtained after a 10-min preincubation with HCTZ and probably fluctuates for some time; (iii) HCTZ, though slowly, completely inhibits

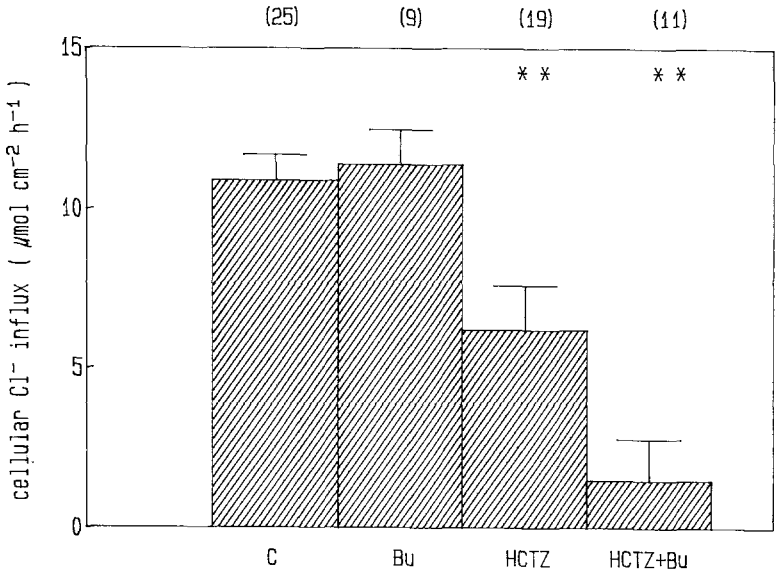

Fig. 5. Cellular Cl^- influx across the apical membrane: effects of bumetanide (*Bu*, 10^{-4} M) and HCTZ (10^{-3} M). Bumetanide was added to the luminal medium only during the measuring time (45 sec), whereas HCTZ was also present in the medium during the 45 min preincubation time. *C* = control. Number of experiments is shown in parentheses. $^{**}P < 0.01$ relative to control.

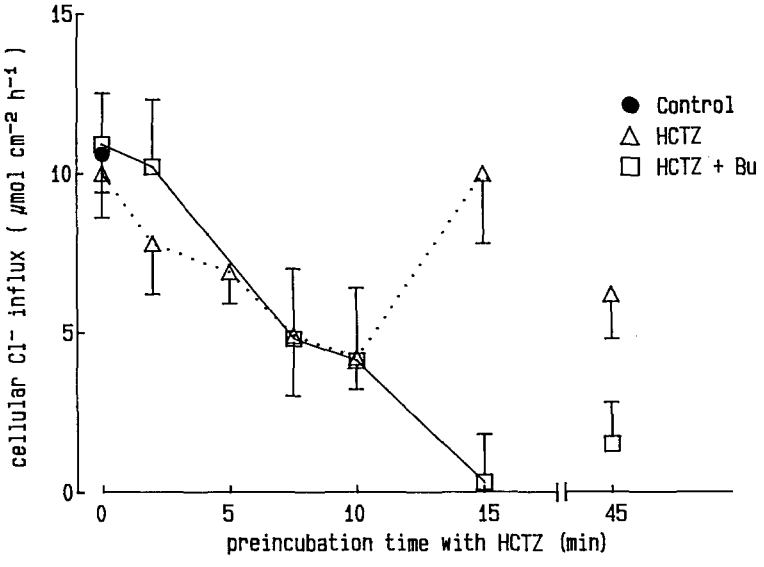

Fig. 6. Cellular Cl^- influx across the apical membrane: time course of HCTZ effects. HCTZ (10^{-3} M) was present during the measuring time (45 sec) and during preincubation periods variable in length from 0 to 45 min. Bumetanide (Bu, 10^{-4} M) was added only during the measuring time. Control: $n = 18$. Treated tissues: $n = 4$–11 for each data point except for points at 5 and 45 min with HCTZ ($n = 26$ and 19, respectively).

the Cl^- influx occuring through the original transport pathway.

Intracellular Cl^- Activity

An original registration of the voltage changes obtained with the conventional and Cl^--selective channels of a theta microelectrode upon cell penetration is reported in Fig. 7; the signal recorded with the Cl^--selective channel (*B*) at steady state corresponds to the apparent chemical potential difference between cell and lumen (membrane potential was automatically subtracted in real time from the measured apparent electrochemical potential difference). The two signals reached stability in this case after 5 and 9 sec, respectively. When signal *B* reached stability, impalement was maintained for a further 22 sec. Entry into the cell is marked by the abrupt voltage change of both traces *A* and *B* and by the voltage deflections, caused by the direct current pulses, which become about three times larger upon cell penetration; return to the baseline is immediate and complete for both traces.

Under control conditions apical membrane potential (V_m) and intracellular Cl^- activity ($a_{i,Cl}$) did not change significantly for the 90-min observation period (Table 1); Cl^- accumulation ratio (intracellu-

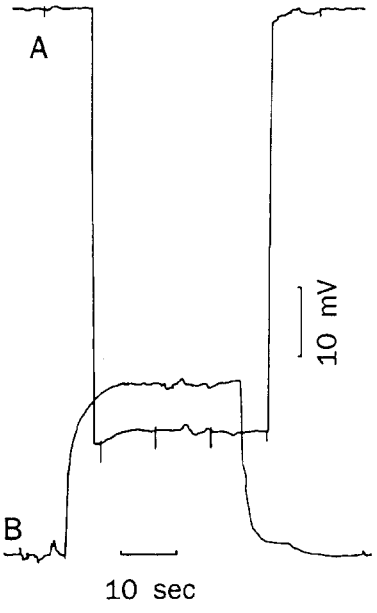

Fig. 7. Registration of the voltage changes obtained with the conventional (*A*) and Cl^--selective (*B*) shanks of a theta-microelectrode upon cell penetration. The recorded signal *B* corresponds only to the apparent chemical potential difference, signal *A* being automatically subtracted in real time. The lag between the voltage changes of the two traces (apparently 5 sec) is due to an artificial displacement of the two traces themselves to avoid overlappings. Voltage deflections every 10 sec are caused by pulses of direct current (20 μA/0.17 cm^2).

lar Cl^- activity measured, compared to that predicted at the electrochemical equilibrium) was higher than three, similar to that previously reported (Cremaschi et al., 1987*b*) (Table 2). Concomitantly, transepithelial potential difference (V_{ms}) was -0.4 ± 0.2 mV (six gallbladders, serosa-mucosa) for all the period.

Possible changes in Cl^- activity, elicited by 10^{-3} M HCTZ, were checked in six gallbladders. After a control period of 30 min, treatment began and was prolonged for 90 min from the beginning of the experiment. Actually Cl^- activity decreased significantly upon HCTZ treatment (Table 1), from about 30 to about 20 mM, the minimum value reached within 15–30 min of treatment; then the activity rose significantly above this minimum to reach a value not significantly different from the control at about 60 min of treatment.

HCTZ caused a slight decrease in the apical membrane potential in the same cells (about 3 mV), already highly significant within 15 min of treatment (Table 1). This depolarization spontaneously receded so as to be not significant at about 60 min of treatment (it was not any more significantly different from its outer control already at 45 min of treatment).

The Cl^- accumulation ratio dropped from 3.8 to a minimum of 2.2 at about 30 min of treatment; then increased again to reach a value not significantly different from the control at the end of the experiment.

In four of these gallbladders the transepithelial potential difference was measured: it was -0.5 ± 0.2 mV during the control period and remained equal to -0.6 ± 0.3 mV at any time during 60 min of treatment. Thus, HCTZ does not significantly affect this parameter.

Finally, the epithelium of five gallbladders was exposed on the luminal side to 10^{-3} M HCTZ and 5×10^{-5} M bumetanide, concomitantly present. The results obtained are again reported in Tables 1 and 2.

The intracellular Cl^- activities dropped significantly within 15 min from about 30 to about 17 mM and remained at a minimum value of 15–16 mM during all of the subsequent treatment periods. All these values were significantly lower than those obtained both in control and HCTZ-treated tissues. The apical membrane potential displayed the same tendency to decrease observed in HCTZ-treated tissues, but the depolarization was significantly larger after 30 min of treatment and persistent for 60 min. In one gallbladder the transepithelial potential difference was measured and proved to be unaffected by the treatment (-0.3 mV in the control period and during the 60-min treatment period). The Cl^- accumulation ratio calculated under control conditions was not significantly different from that obtained in the previous set of experiments. However, upon treatment, it already decreased significantly to a larger extent within 15 min and then persisted equal to 1.5–1.6 for 60 min of treatment, always significantly lower than the corresponding values measured upon treatment with HCTZ alone.

In conclusion, these experiments showed that HCTZ reduces intracellular Cl^- activity and accumulation ratio to minima apparently reached within 15–30 min, with a subsequent increase of the two parameters until at least 60 min of treatment. Starting from 15 min of treatment, the fraction of Cl^- still accumulated intracellularly, with an activity over 15 mM and a ratio greater than 1.5, is related to a new, bumetanide-sensitive, transport process. This latter fraction is absent under control conditions (Cremaschi et al., 1987*b*), appears after treatment and increases with time in two steps, the first around 15 min and the second around 60 min of treatment. Thus, the actual inhibition due to HCTZ is masked by this component and is much more substantial and persistent, in complete agreement with the gravimetric and radiochemical measurements.

Table 1. Time course of apical membrane potential (V_m) and intracellular Cl^- activity ($a_{i,Cl}$) under different experimental conditions

Experimental condition		Control (15–30 min)	Treatment (in *b*, *c* conditions)			
			(45 min)	(60 min)	(75 min)	(90 min)
V_m (mV)	a) control	−62.2 ± 1.0 (28)	−61.8 ± 0.5 (35)	−60.5 ± 0.3 (37)	−60.6 ± 0.6 (32)	−61.2 ± 0.7 (31)
	b) HCTZ	−62.0 ± 0.5 (34)	−59.1 ± 0.7** (29)	−58.9 ± 0.7** (37)	−59.3 ± 0.6** (30)	−61.4 ± 0.7 (28)
	c) HCTZ + Bu	−61.5 ± 0.3 (35)	−59.0 ± 0.6** (24)	−56.7 ± 0.5** (21)	−57.6 ± 0.6** (21)	−56.1 ± 0.8** (20)
P	b-a	NS	***	*	NS	NS
	c-a	NS	***	***	***	***
	c-b	NS	NS	*	*	***
$a_{i,Cl}$ (mM)	a) control	29.4 ± 1.3 (28)	29.4 ± 1.1 (35)	28.2 ± 1.0 (37)	30.0 ± 1.3 (32)	30.0 ± 1.4 (31)
	b) HCTZ	30.9 ± 1.2 (34)	22.3 ± 1.2** (29)	20.1 ± 0.8** (37)	20.4 ± 1.3** (30)	29.3 ± 1.6 (28)
	c) HCTZ + Bu	30.3 ± 1.0 (35)	17.5 ± 0.9** (24)	16.2 ± 0.7** (21)	15.9 ± 0.7** (21)	15.3 ± 0.7** (20)
P	b-a	NS	***	***	***	NS
	c-a	NS	***	***	***	***
	c-b	NS	***	***	***	***

Treatments (luminal 10^{-3} M HCTZ, alone or with 5×10^{-5} M bumetanide, Bu) started after 30 min from the beginning of the experiment and were prolonged for 90 min. Results are presented as means ± SE with the number of impalements in parenthesis; they were obtained from 6, 6 and 5 gallbladders for a, b and c conditions, respectively. During treatments cells were punctured in the period included between 5 min before and after the time indicated. *P* = statistical probability; NS = not significant; $*P < 0.05$, $*** P < 0.01$ (compared to the outer control value at the same time); $**P < 0.01$ (compared to the respective inner control value at 15–30 min).

Table 2. Time course of the intracellular Cl^- accumulation ratio, in relation to intracellular Cl^- activity predicted at the electrochemical equilibrium ($a_{i,Cl}/a_{i,Cleq}$), under different experimental conditions

Experimental condition		Control (15–30 min)	Treatment (in *b*, *c* conditions)			
			(45 min)	(60 min)	(75 min)	(90 min)
$a_{i,Cl}$ $a_{i,Cleq}$	a) control	3.6 ± 0.1 (28)	3.6 ± 0.2 (35)	3.2 ± 0.1** (37)	3.3 ± 0.1* (32)	3.6 ± 0.2 (31)
	b) HCTZ	3.8 ± 0.2 (34)	2.4 ± 0.1** (29)	2.2 ± 0.1** (37)	2.2 ± 0.1** (30)	3.5 ± 0.2 (28)
	c) HCTZ + Bu	3.6 ± 0.2 (35)	1.9 ± 0.1** (24)	1.6 ± 0.1** (21)	1.6 ± 0.1** (21)	1.5 ± 0.1** (20)
P	b-a	NS	***	***	***	NS
	c-a	NS	***	***	***	***
	c-b	NS	***	***	***	***

The ratio was calculated from each impalement, on the basis of the individual data whose means are reported in Table 1. Further information is in Table 1. $*P < 0.05$. Compared to the respective inner control value at 15–30 min.

Intracellular K^+ Activity

An original registration of the voltage changes obtained with the conventional and K^+-selective channels of a theta microelectrode upon penetration is reported in Fig. 8; the signal recorded with the K^+-sensitive shank (trace *B*) at steady state corresponds to the apparent chemical potential difference between lumen and cell as in the case of the Cl^--sensitive shank. During this impalement both signals reached stability within 3–4 sec. When stability was reached, impalement was maintained for a further 19–20 sec. Entry into the cell is marked by the sudden voltage change of both traces *A* and *B* and by

Table 3. Time course of intracellular K^+ activity ($a_{i,K}$) under different experimental conditions

Experimental condition		Control (15–30 min)	Treatment (in *b*, *c* conditions)			
			(45 min)	(60 min)	(75 min)	(90 min)
$a_{i,K}$ (mM)	a) control	78.5 ± 2.1 (45)	76.5 ± 1.9 (38)	79.1 ± 2.0 (38)	79.3 ± 2.0 (36)	76.4 ± 1.9 (24)
	b) HCTZ	79.9 ± 2.6 (47)	86.4 ± 2.3 (42)	83.6 ± 2.5 (39)	93.6 ± 2.0** (46)	93.7 ± 1.7** (42)
	c) HCTZ + Bu	79.0 ± 2.0 (42)	77.6 ± 2.0 (35)	78.0 ± 1.8 (30)	75.0 ± 1.8 (32)	75.0 ± 2.0 (30)
P	b-a	NS	***	NS	***	***
	c-a	NS	NS	NS	NS	NS
	c-b	NS	***	NS	***	***

Results were obtained from six control gallbladders, six gallbladders treated with HCTZ (10^{-3} M) and four treated with HCTZ (10^{-3} M) and bumetanide (5×10^{-5} M; Bu). Further information is in Tables 1 and 2.

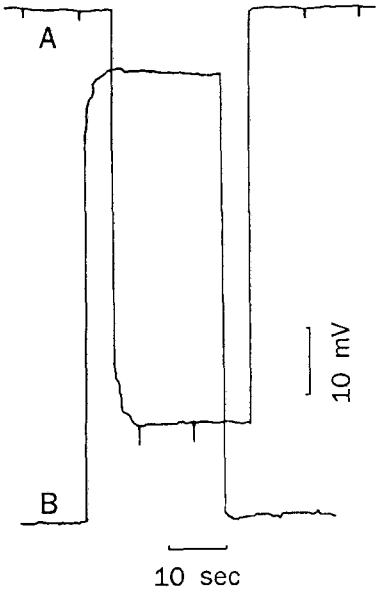

Fig. 8. Registration of the voltage changes obtained with the conventional (*A*) and K^+-selective (*B*) channels of a theta-microelectrode upon cell penetration. The recorded signal *B* corresponds only to the apparent chemical potential difference, signal *A* being automatically subtracted in real time. Further information is given in the legend of Fig. 7.

the voltage deflections (caused by the direct current pulses of 20 μA), which become about three times larger, compared to those obtained with the microelectrode placed out of the cell; return to the baseline is immediate and complete for both traces.

Under control conditions intracellular K^+ activity was stable for at least 90 min (Table 3). Conversely, after luminal treatment with 10^{-3} M HCTZ, it increased over a period of time, compared with the control values (Table 3). The first increase apparently occurred within 15 min of treatment; it was not significantly different from its inner control before treatment ($P \simeq 0.07$), but reached a $P < 0.01$ compared to the external control at the same time. The maximal increase was observed after a 45-min treatment and persisted until at least 60 min of treatment.

If 5×10^{-5} M bumetanide was dissolved in the luminal medium with 10^{-3} M HCTZ, the increase in intracellular K^+ activity was completely eliminated (K^+ activity did not differ significantly compared to control values: Table 3).

In these groups of experiments the time courses of the apical membrane potential under the different experimental conditions were similar to those reported in Table 1.

Discussion

We have previously shown that under control conditions, in the presence of bicarbonate in the bathing salines, about half the NaCl transport is sustained by a Na^+/H^+, Cl^-/HCO_3^- double exchange, whereas the complementary half is related to a Na^+-Cl^- symport on single carrier (*see* Introduction). The results reported here confirm that the symport is not affected by bumetanide; moreover, they show that it is inhibited by HCTZ. On a first approach it may appear that only a fraction of it is sensitive to HCTZ. However, all the results obtained by measuring the different parameters taken into account indicate that the apparently remaining fraction is actually a new, different symport, sensitive to bumetanide, activated as a consequence of inhibition of the former. Thereby, the entire original symport proves sensitive to HCTZ. In principle, 10^{-4} M bu-

metanide can also inhibit the anion exchanger, but the absence of exogenous bicarbonate and the presence of acetazolamide in the incubation salines, the independence of SITS and the relationship with K^+ are all factors indicating that the residual transport cannot be related to a homeostatically activated Cl^-/HCO_3^- exchange, but to a Na^+-Cl^-, Na^+-K^+-$2Cl^-$ symport system.

Among the tested parameters Cl^- accumulation ratio ($a_{i,Cl}/a_{i,Cleq}$) deserves particular discussion. It has a physiological meaning, in spite of the absence of Cl^- conductance in the apical membrane under control conditions (Cremaschi & Hénin, 1975; Hénin & Cremaschi, 1975; Cremaschi & Meyer, 1982; Petersen, Wehner & Winterhager, 1990), since a small Cl^- conductance is present at the basolateral membrane (Cremaschi & Hénin, 1975). Moreover, a detailed analysis (*in preparation*) of the slight, but significant depolarization observed upon HCTZ treatment indicates that it is due to the progressive opening of an apical Cl^- conductance; in this way, Cl^- accumulated in the cell by either symport form, one progressively inhibited, the other activated, in part backdiffuses to the lumen and depolarizes V_m. This Cl^- conductance is more substantial than might appear on the basis of the slight depolarization measured, since the effects of changes in the apical electromotive force are attenuated by: (i) the shunting operated by the leaky paracellular pathways, (ii) the increase in intracellular K^+ activity, (iii) the opening of apical K^+ channels elicited by HCTZ, also in agreement with Duffey and Frizzell (1984).[2] The tendency to increase (1–2 μmol cm^{-2} hr^{-1}) of the SCN^--insensitive fraction of the influx, measured radiochemically, is likely to be due to the effects of the new Cl^- conductance.

The significantly larger depolarization, observed upon HCTZ and bumetanide treatment, may be related to the decrease in intracellular K^+ activity that follows this treatment. Cl^- in this case is likely to be passively distributed at the electrochemical equilibrium, in spite of the fact that the Cl^- accumulation ratio apparently decreases only to about 1.5. In fact, under these conditions: (i) the ratio 1.5–1.6 is steady in time, (ii) transepithelial NaCl transport is abolished, (iii) interference by cellular phosphate and organic anions with Cl^- activity measurements is likely to be present owing to the lower cellular Cl^- activity, all the more so as the probable cell shrinkage under these conditions causes an increase in the cellular activity of these intracellular anions.[3]

[2] In the paper in preparation the apical Cl^- conductance will be shown also to contribute partially (together with the main inhibitory action on the Na^+-Cl^- symport) to the dissipation of intracellular Cl^- accumulation elicited by HCTZ treatment.

Features of the Action of HCTZ

From the dose-response relationship obtained with fluid transport measurements, the apparent half-maximum effective dose IC_{50} and the apparent dose giving maximum inhibition proved to be 2×10^{-4} M and about 10^{-3} M, respectively. These values appear to be 8–10 times larger than the corresponding values determined in fish urinary bladder, where the half-maximum and maximum inhibition are obtained with $2–5 \times 10^{-5}$ M and about 10^{-4} M concentrations, respectively (Stokes, 1984). However, as for gallbladder, it is worth noting that the activation of the new, bumetanide-sensitive symport makes the dose-response relationship complex and approximate, and possibly simulates lower sensitivity. Moreover, an inspection of the bathing salines used for gallbladder and urinary bladder has indicated as the main difference the presence of sulfate and phosphate in the former case (17 mM in total). Preliminary experiments we are now carrying out indicate that in gallbladder too, maximum inhibition with HCTZ is already reached at a concentration of 10^{-4} M if sulfate and phosphate are eliminated from the bathing saline.

[3] In one group of animals intracellular Cl^- activity under control conditions was lower than that reported in Table 1 (about 18 mM), with a Cl^- accumulation ratio of about 2. HCTZ effects in this case were quantitatively more attenuated but qualitatively roughly similar to those reported above (gradual V_m depolarization, intracellular Cl^- activity and accumulation ratio reduced to about 12 mM and 1.3 within 30 min with subsequent gradual increase to values of about 17 mM and 1.7 at 60 min of treatment). However, after treatment with HCTZ and bumetanide concomitantly present, intracellular Cl^- activity did not further decrease: it was significantly lower than that obtained with HCTZ alone only at 60 min of treatment. In spite of this, in relation to the greater depolarization obtained by this treatment, the calculated Cl^- accumulation ratio dropped to about 1.3 within 15 min and remained so permanently. In conclusion, HCTZ-inhibited Cl^- entry into the cell and a bumetanide-sensitive transport was elicited also in this case.

The fact that intracellular Cl^- activity did not decrease for nearly all the observation period in the presence of bumetanide and HCTZ, as compared to HCTZ alone, necessitated a balance between the decrease in intracellular Cl^- activity caused by bumetanide *per se* and a conductive Cl^- uptake, possibly aided by a simultaneous decrease in the rate of the basolateral Cl^- export mechanism. The substantial apical Cl^- conductance discussed above, with the small basolateral conductance of the anion, should be sufficient at the lower intracellular Cl^- activities observed in these experiments, to effect the balance, with transient conductive Cl^- uptakes, and rearrange Cl^- distribution in relation to the lower membrane potential. Moreover, as discussed above, under these extreme treatment conditions intracellular Cl^- activity was likely to be overestimated for the increased interference of the other intracellular anions; thus, its actual value, in the presence of bumetanide and HCTZ, was probably lower than that apparently measured.

Analysis of the inhibition time course performed on the Cl^- influx shows that HCTZ does not immediately inhibit the symport, unlike SCN^- (Cremaschi et al., 1987*a*). It takes some minutes before the first significant decrease in the Cl^- influx is observed and it is only after 10–15 min from the beginning of treatment that inhibition is completed. HCTZ action is not immediate in fish urinary bladder either, though it is faster than in gallbladder; the response begins within 5–30 sec and is usually complete within 2–3 min (Stokes, 1984). Moreover, metolazone, which binds to the same receptor as the thiazide type diuretics (Beaumont, Vaughn & Fanestil, 1988), and is usually included in the thiazide class because of its apparently similar action (Gilman, Goodman & Gilman, 1980), is even slower to commence and complete its action in urinary bladder (Stokes, 1984).

This delayed, slow inhibition strongly suggests that inhibition is not the outcome of direct HCTZ action on the carrier, but is mediated by metabolic effects or some cytoplasmic factors, all the more so as HCTZ is poorly water soluble and maybe enters the cell.

The possibility of inhibitory effects on metabolism[4] and/or Na^+/K^+-ATPase is ruled out by the observed constancy, or even increase, of $a_{a,K}$ (Table 3) and by the constancy, or tendency to decrease, of $a_{i,Na}$ (10.7 ± 1.0 mM, $n = 6$, after 20 min treatment, as against the control value of 11.9 ± 0.7 mM, $n = 8$; *unpublished results*).

The possibility that HCTZ operates by increasing intracellular 3′,5′ cyclic adenosine monophosphate (cAMP) system is suggested by the inhibition of phosphodiesterase by some thiazides (Moore, 1968; Vulliemoz, Verosky & Triner, 1980). However, on one side, this inhibition was obtained only at very high doses; on the other side, in gallbladder epithelium cAMP certainly inhibits the neutral Na^+-Cl^- coupled entry (Frizzell et al., 1975) and in particular the double ion exchange (Petersen, Wehner & Winterhager, 1985; Reuss & Petersen, 1985; Reuss, 1987), but its possible action on the Na^+-Cl^- symport we have shown is so far uncertain (Petersen et al., 1990). In agreement, exposures of fish urinary bladder to 8-Br-cAMP had no effect on Na^+-Cl^- symport (Stokes, 1984).

Finally, the complete inhibitory effect on symport obtained with 5×10^{-4} M HCTZ in a few tens of seconds in the isolated apical membrane vesicles of rabbit gallbladder (Meyer et al., 1990) indicates that the site of action is located in the membrane and, since these experiments were carried out in the absence of ATP, GTP or Ca^{2+} gradients, the action cannot be mediated by intramembrane modulators like G-proteins or calmodulin. Hence the site of action seems to be located on the carrier. Two hypotheses can be put forward to explain why inhibition time is a few tens of seconds in the case of vesicles and several minutes in the case of the cell: (i) the site of action might be on the inner side of the membrane and, in this case, HCTZ would have to cross the membrane to bind and the concentration sufficient to inhibit would, of course, be reached in a shorter time in the smaller volume of the vesicles; (ii) the sensitivity of the carrier to the drug and the time course of the action of HCTZ might be regulated by a control unit, which might be eliminated during the apical membrane isolation procedures. In agreement with the latter hypothesis it has recently been shown that renal thiazide receptors undergo rapid, reversible regulation and that controlling mechanisms are dependent on cellular ATP levels (Beaumont et al., 1989).

[4] In principle this possibility has to be taken into account since many diuretics inhibit metabolic processes (Eknoyan et al., 1975; Manuel & Weiner, 1976, 1977; Weller & Borondy, 1976).

Bumetanide-Sensitive Na^+-Cl^- Symport

The bumetanide-sensitive symport is already activated during the first 15 min incubation with HCTZ; then it gradually increases to completely replace the original HCTZ-sensitive symport with time. The parameters measured with better temporal resolution (cellular Cl^- influx and accumulation) also show that the bumetanide-sensitive symport seems to appear at about 15 min of treatment and then to display a second increase at around 60 min of treatment.

Within 15–30 min of treatment with HCTZ, the intracellular K^+ activity also tends to increase, but a significant bumetanide-sensitive K^+ activity rise is observed only in the last two experiment periods. Hence the time course is not completely corresponding to that of changes in Cl^- activity, but it may be that the new symport is better defined as a Na^+-Cl^-, Na^+-K^+-$2Cl^-$ symport system, whose capacity to transport K^+ perhaps increases with time.

The signal for its activation may be the cell shrinkage, probably produced by the inhibition of NaCl entry into the cell, caused by HCTZ. The new symport allows a regulatory volume increase by again introducing osmotically active solutes into the cell. Stimulation of Na^+-K^+-$2Cl^-$ symport, owing to cell shrinkage, has been extensively observed in a variety of transporting epithelia such as thick ascending limb, lacrimal gland, intestinal glands, trachea, etc. (Lang, Volkl & Haussinger, 1990). In rabbit gallbladder, the symport certainly also counteracts inhibition of the transepithelial transport of

Na^+ and fluid, thereby being homeostatic for both cell and epithelial functions.

The Three Different Forms of Neutral Na^+-Cl^- Coupled Transport

During the last 20 years the nature of the neutral Na^+-Cl^- coupled transport has gradually proved to be complex. Transport proved to be sustained either by a Na^+/H^+, Cl^-/HCO_3^- double exchange (Murer, Hopfer & Kinne, 1976; Liedtke & Hopfer, 1977, 1982*a,b*; Aronson & Seifter, 1984), a bumetanide-sensitive Na^+-Cl^-,Na^+-K^+-$2Cl^-$ symport system (Geck et al., 1980; Ellory et al., 1982; Eveloff & Kinne, 1983; Frizzell & Field, 1984; Greger, 1984; Alvo, Calamia & Evaloff, 1985) or a HCTZ-sensitive Na^+-Cl^- symport (Duffey & Frizzell, 1984; Stokes, 1984; Velasquez, 1987). In the nephron these three forms are all present, though distributed in succession, the former in the proximal tubule, the second in the thick ascending limb, the third in distal tubule.

As for gallbladder, the dual ion exchange was first discovered as a result of the work of Heintze and Petersen (1980), Baerentsen, Giraldez and Zeuthen (1983) and Reuss (1984) on the guinea-pig and *Necturus*. However, the importance of the dual ion exchange in transepithelial transport was challenged by Ericson and Spring (1982) and Spring (1984), who showed that in *Necturus* under normal conditions transepithelial transport is sustained by the symport, inhibited by bumetanide and apparently insensitive to K^+, whereas the double exchange seems to be activated only in relation to regulatory volume increase, caused by exposure of the epithelium to hypertonic saline.

Similar results were obtained by Davis and Finn (1985), again in *Necturus;* from their experiments the bumetanide-sensitive symport proved K^+-dependent and the countertransport pathways seemed to be activated when the Na^+-K^+-$2Cl^-$ cotransporter was inhibited. According to these authors, both transport mechanisms appeared also operative under control conditions since transepithelial fluid transport was inhibited not only by bumetanide, but also by amiloride and dypiridamole (an inhibitor of Cl^-/HCO_3^- exchange).

In the data of Davis and Finn many observations suggest that the countertransport and cotransport systems are not actually concomitantly active and that under control conditions bumetanide at high doses inhibited the Cl^-/HCO_3^- exchange rather than the Na^+-K^+-$2Cl^-$ symporter. In fact: (i) bumetanide and amiloride each inhibited transepithelial fluid transport almost completely instead of inhibiting complementary fractions only; (ii) removal of K^+ under control conditions had no effect on cell volume; only the ouabain induced cell swelling was K^+ dependent; and (iii) the rate of K^+-dependent fluid influx across the apical membrane during ouabain-induced cell swelling was much lower than the rate of transepithelial fluid transport under control conditions.

All this, together with the observation that removal of luminal K^+ does not affect the intracellular Cl^- activity (Reuss, 1984), rather suggests that under control conditions the double ion exchange is at least dominant in *Necturus*. However, it is maintained that, taking the data of the authors quoted above as a whole, in *Necturus* both double ion exchange and Na^+-Cl^-, Na^+-K^+-$2Cl^-$, bumetanide-sensitive symporter seem to be present and involved at times in transepithelial transport, at times in volume regulation and at times in both processes, probably depending on animal and experiment conditions.

In the case of rabbit gallbladder we have shown that the double ion exchange clearly operates under control conditions and is involved in transepithelial transport (Cremaschi et al., 1983, 1987*a*), in complete agreement with the results of Heintze and Petersen (1980), Baerentsen et al. (1983) and Reuss (1984) for guinea-pig and *Necturus*. However, we have also produced evidence that a K^+-independent, bumetanide-insensitive symport operates in parallel to the double ion exchange (Cremaschi et al., 1983, 1987*a,b*; Meyer et al., 1990); we now know that this symport is HCTZ-sensitive and apparently similar to that found in fish urinary bladder and mammal distal tubule. This paper, moreover, shows that the Na^+-Cl^-, Na^+-K^+-$2Cl^-$, bumetanide-sensitive symport system is also latently present and becomes activated when the other two transport systems are both inhibited; it certainly operates homeostatically for transepithelial transport and probably for cell volume regulation. The latter finding is in agreement with Ericson and Spring (1982), Spring (1984) and Davis and Finn (1985), thereby confirming that "the proportion of NaCl transported by the different mechanisms varies with the conditions."

We previously discovered the presence of double ion exchange and of two different Na^+-Cl^- symports in rabbit by analyzing the kinetics of Cl^- entry at the apical membrane; one of the two symports was only activated when luminal Cl^- concentration was low and was completely silent under control conditions; though its presence was certain, its definition was uncertain (Cremaschi et al., 1983). On the basis of the present results it was likely to correspond to the Na^+-K^+-$2Cl^-$ symport, but validation of this hypothesis requires further experiments.

In conclusion, it is noteworthy that the three different forms of neutral Na^+-Cl^- coupled transport detected to date seem to be present all together in the same membrane. The physiological meaning of the simultaneous presence of double ion exchange and HCTZ-sensitive symport under control conditions has been previously discussed and associated with the very large-scale transport of Na^+ and fluid effected by rabbit gallbladder (Cremaschi et al., 1987*a*) compared with *Necturus*. The third system clearly occurs under particular conditions as a homeostatic factor.

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