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Hydrochlorothiazide Action on the Apical Cl^-, Ca^{2+} and K^+ Conductances in Rabbit Gallbladder Epithelium. Presence of an Apamin-sensitive, Ca^{2+}-activated K^+ Conductance

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Abstract. In the rabbit gallbladder epithelium, hydrochlorothiazide (HCTZ) was shown to inhibit the transepithelial NaCl transport and the apical Na^+-Cl^- symport, to depolarize the apical membrane potential and to enhance the cell-to-lumen Cl^- backflux (radiochemically measured), this increase being SITS-sensitive. To better investigate the causes of the depolarization and the Cl^- backflux increase, cells were punctured with conventional microelectrodes on the luminal side (incubation in bicarbonate-free saline at 27°C) and the apical membrane potential (V_m) was studied either with prolonged single impalements or with a set of short multiple impalements. The maximal depolarization was of 3–4 mV and was reached with 2.5×10^{-4} M HCTZ. It was significantly enhanced by reducing luminal Cl^- concentration to 30 mM; it was abolished by SCN^-, furosemide, SITS; it was insensitive to DPC. SITS converted the depolarization into a hyperpolarization of about 4 mV; this latter was apamin, nifedipine and verapamil sensitive. It was concluded that HCTZ concomitantly opens apical Cl^- and (probably) Ca^{2+} conductances and, indirectly, a Ca^{2+}-sensitive, apamin inhibitable K^+ conductance: since the intracellular Cl^- activity is maintained above the value predicted at the electrochemical equilibrium, the opening of the apical Cl^- conductance depolarizes V_m and enhances Cl^- backflux. In the presence of apamin or verapamil, to avoid the hyperpolarizing effects due to HCTZ, the depolarization elicited by this drug was fully developed (7–10 mV) and proved to be Ca^{2+} insensitive. On this basis and measuring the transepithelial resistance and the apical/basolateral resistance ratio, the Cl^- conductance opened by HCTZ has been estimated and the Cl^- backflux increase calculated: it proved to be in the order of that observed radiochemically. The importance of this Cl^- leak to the lumen in the overall inhibition of the transepithelial NaCl transport by HCTZ has been evaluated.

Key words: NaCl transport — SITS — SCN^- — DPC — Furosemide — Verapamil

Introduction

We have previously shown that hydrochlorothiazide (HCTZ) inhibits the bumetanide and K^+ insensitive Na^+-Cl^- symport present in the apical membrane of rabbit gallbladder epithelium (Cremaschi et al., 1983, 1987*a, b*, 1992; Meyer et al., 1990); maximal effects were reached with a 10^{-3} M concentration (Cremaschi et al., 1992). Concomitantly the drug decreased the apical membrane potential by about 3 mV (Cremaschi et al., 1992).

This depolarization is in some way unexpected, inasmuch as in fish urinary bladder HCTZ treatment inhibits Na^+-Cl^- symport (Duffey & Frizzell, 1984; Stokes, 1984) and hyperpolarizes the apical membrane, the hyperpolarization being generated by an increase in K^+ conductance (Duffey & Frizzell, 1984).

In accordance with the inhibition of the transepithelial NaCl transport (Cremaschi et al., 1992) the depolarization observed in gallbladder might arise from the appearance of an apical Cl^- conductance (absent under basal condition: for a review *see* Cremaschi & Porta, 1992) which allows the Cl^- accumulated in the cell by the symport to escape back to the lumen. In this case, inhibition of transport should be ascribed concomitantly to the symport inhibition and the Cl^- conductance opening.

Hence, we have analyzed the depolarization causes

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to gain further insight into the mechanism of HCTZ action and shed some light on the apparent contradiction between results obtained in gallbladder and fish urinary bladder. Experiments were performed in bicarbonate-free saline to silence the apical Cl^-/HCO_3^- exchange and to allow the symport to be the only supporter of NaCl transport (Cremaschi et al., 1987*a, b*).

Results obtained with conventional microelectrodes and the use of ion channel inhibitors are presented; further research with radiochemical techniques and with Cl^-- and Na^+-selective *theta*-microelectrodes has been accomplished (Cremaschi & Porta, 1994; *in preparation*).

Preliminary results have been presented in abstract form (Cremaschi et al., 1993; Vallin et al., 1994).

Materials and Methods

Male New Zealand rabbits (body weight about 3 Kg) were killed by cervical dislocation and the excised gallbladders washed free of bile with a bicarbonate-free solution (*see* "Salines").

Electrophysiological Measurements

Apical membrane potentials were measured with conventional microelectrodes filled with 0.5 M KCl (tip diameter less than 0.3 μm, tip resistance from 50 to 70 MΩ, tip potentials less than 3 mV; glass fiber in the tubing). Microelectrodes were connected through a high impedance electrometer (model FD223, World Precision Instruments, New Haven, CT) to a strip chart recorder (PM 8262 Xt Recorder, Philips, Eindhoven, The Netherlands). Short pulses of direct current (200 msec, 20 μA) generated by a SD5 stimulator (Grass Instruments, Quincy, MA) were passed through the exposed tissue (0.17 cm^2) every 20 sec by means of Ag/AgCl electrodes in order to check microelectrode entrance into the cell from the voltage deflections. Gallbladders, opened flat, were mounted so as to separate an upper mucosal from a lower serosal Lucite chamber, both perfused (12 ml/min) by gravity and kept inclined 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) to maintain the reservoir level constant. The time for the renewal of the fluid on the epithelium was 4 sec. Single impalements (prolonged for 21 min, having reached stability) or sets of multiple short impalements (each about 20-sec long, having reached stability) were used to check membrane potentials. Multiple short impalements, much easier to obtain in the slender cells of the rabbit gallbladder epithelium, were used to rapidly check the effects of the treatment or, when following the development of the effect in the time was not important. General criteria for impalement validation were: (i) the potential difference measured had to change abruptly, as well as the voltage deflections caused by the d.c. pulses which should increase, (ii) after the stability of the p.d. had been reached the signal had to remain constant for at least 20 sec, (iii) finally the p.d. had to return rapidly to the baseline on leaving the cell.

The general protocol used for single impalements was as follows: (*a*) having reached stability, 1-min observation under control conditions, (*b*) treatment with the inhibitor for 10 min, (*c*) back to control conditions for 10 min. The protocol followed for multiple impalements was: (*a*) 30 min under control conditions with a set of impalements taken between 20 and 30 min (control), (*b*) treatment for 20 min with a set of impalements between 10 and 20 min (treatment), (*c*) control conditions for 40 min with a set of impalements between 30 and 40 min (control).

The transepithelial potential difference (V_{ms}) was measured by piercing the basolateral membrane with the microelectrode tip. The crossing was acknowledged by an abrupt return of the measured voltage toward zero. The voltage deflections across the apical (ΔV_m) and basolateral (ΔV_s) membranes were calculated from the voltage deflections elicited by the current passage, measured just before entering apically the cell or in the cell or out of the cell basolaterally. On this basis, the apical/basolateral resistance ratio (R_m/R_s) and the transepithelial resistance (R_{ep}) were also calculated.

The change in junction potential across the boundary formed by the saline bath/0.5 M KCl agar bridge (connected to the reference calomel electrode), when a low Cl^--saline was substituted for the high Cl^--saline in the lumen, was corrected as reported.[1]

Salines

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, 2.7 HPO_4^{2-}, 0.7 $H_2PO_4^-$; pH 7.4. Inhibitors were added to the solution without any vehicle (the change in osmolality was negligible). SCN^- (25 mM) was substituted for 12.5 mM SO_4^{2-} and mannitol. HCTZ was directly dissolved in the saline, although solubilization by this method was slow: the final concentration was checked with a spectrophotometer (Lambda 5, Perkin Elmer, Norwalk, CT) at the wavelength of 226 nm. In the low Cl^--saline 95.3 mM Cl^- (introduced as NaCl) were replaced by cyclamate. Salines were bubbled with 100% O_2 and kept at 27 ± 1°C to maintain the isolated tissue viable and stable over time.

Materials

HCTZ, SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate), furosemide, apamin, A23187 ionophore, verapamil, nifedipine, formaldehyde were purchased from Sigma (St. Louis, MO); 5-chloro-2,4-disulfamylaniline and DPC (N-phenylanthranilic acid or diphenylamine-2-carboxylic acid) from Aldrich Chemical (Milwaukee), SCN^- from Carlo Erba-Farmitalia (Milan, Italy). All other reagents used were of AR grade.

Statistics

The results were presented as means ± SE with the number of impalements in parentheses. Statistical probability was analyzed with the Student's *t*-test; where possible, paired-data analysis was used. The

[1] Glass tip potential changes, read against a microelectrode with broken tip, were nil, changing to a low Cl^- saline; junction potential changes at the open tip of the microelectrodes were read on microelectrodes with broken tip against a Nernstian Ag/AgCl electrode (+3.0 ± 0.1 mV, $n = 16$); junction potential changes of microelectrode and reference bridge collectives were determined with a microelectrode *vs.* bridge setup (–2.9 ± 0.2 mV, n = 17). The subtraction to the latter value of the junction potential change value for only the microelectrode gave the junction potential change to be ascribed to the bridge alone (–5.9 ± 0.2 mV, n = 17). Only a fraction of this hyperpolarizing value was recorded during impalement, when the low Cl^- saline was introduced into the lumen, if the cell apical membrane concomitantly depolarized. The actual membrane depolarization was in this way singled out. A similar correction was applied to the recorded V_{ms} changes.

Table 1. Effect of HCTZ or formaldheyde (FA) or 5-chloro-2,4-disulfamylaniline (CDSA) on the apical membrane potential V_m (multiple impalements)

Experimental condition	Time (min):	Control	Treatment in b, c, d		Control		Δ_{t-c}
		20–30	30–40	40–50	50–80	80–90	
(a) Control		−63.7 ± 0.8 (31)		−62.8 ± 0.5^{ns} (41)		−62.5 ± 0.6^{ns} (45)	+0.9 ± 0.9 (36)
(b) HCTZ		−63.1 ± 0.3 (110)		−59.3 ± 0.3* (111)		−63.1 ± 0.3^{ns} (92)	+3.8 ± 0.4$^{\cdot}$ (110)
	V_m (mV)						
(c) FA		−62.1 ± 0.5 (21)		−62.2 ± 0.6^{ns} (21)		−62.8 ± 0.6^{ns} (22)	−0.1 ± 0.8 (21)
(d) CDSA		−62.6 ± 0.5 (26)		−63.1 ± 0.4^{ns} (30)		−62.5 ± 0.5^{ns} (26)	−0.5 ± 0.6 (28)

Time: minutes after the beginning of the experiment. Results presented as means ± SE with the number of impalements in parentheses. Number of gallbladders: 4, 15, 2, 2 respectively for *a, b, c, d* conditions. Δ_{t-c} = difference between mean values obtained under treatment (40–50 min) and control (20–30 min) conditions. HCTZ: 2.5×10^{-4} M; FA: 10^{-4} M; CDSA: 10^{-4} M. Treatment on the luminal side only.
* = $P < 0.01$, either compared to the respective inner (20–30 min) or outer (40–50 min) control.
ns = not significant, either compared to the respective inner (20–30 min) or outer (40–50 or 80–90 min) control.
$^{\cdot}$ = $P < 0.01$ compared to zero.

standard error of the difference of two means (Δ_{t-c}), obtained with unpaired data, was calculated with the formula given.[2]

Results

HCTZ Effects on the Apical Membrane Potential

When multiple short impalements were performed for 90 min in gallbladder epithelial cells maintained under control conditions, the apical membrane potential (V_m) proved to be stable over this period (sets of impalements taken at 20–30, 40–50, 80–90 min after the experiment start; Table 1). Experiments prolonged for 150 min gave the same results (*data not shown*). Luminal treatment with 2.5×10^{-4} M HCTZ between 30 and 50 min produced a depolarization of 3.8 mV, measured with a set of impalements at 40–50 min, i.e., 10–20 min after the treatment start; the effect was completely reversible (Table 1). Depolarization was not significantly different from that previously observed at the same time with a 10^{-3} M concentration of the drug (2.9 mV; Cremaschi et al., 1992); thus, apparently 2.5×10^{-4} M HCTZ already produced maximal effects on the membrane potential, although it cannot be ruled out that the effects were only nearly maximal, considering standard error extents compared to the total value of the depolarizations.

The dissolved HCTZ hydrolyzes with time to produce formaldehyde (FA) and 5-chloro-2,4-disulfamylaniline (CDSA) (Deppeler, 1981). Under our conditions of use, hydrolysis should be present but minimal; nevertheless, we checked the effects of CDSA and FA at 10^{-4} M (supposing 40% HCTZ hydrolyzed). Table 1 shows that the two drugs have no effect. Hence the depolarization observed at HCTZ treatment must be ascribed to the intact HCTZ.

Impalements in a single cell, prolonged for 21 min after stability was reached, were steady over this period under control conditions, in spite of the possible mechanical perturbations elicited by the saline renewal at 1 and 11 min. However, if, during an impalement, after a 1-min control, 2.5×10^{-4} M HCTZ was added to the luminal saline for 10 min, a depolarization was observed after a 20-sec latency; at 30 sec it reached an already significant 0.7 mV value and after 6 min started to increase to reach a maximum (about 3 mV) at 9–10 min; the effect was progressively and completely reversible on restoring the control saline. The depolarization is statistically analyzed in Fig. 1. Its maximal value is not significantly different from that obtained by multiple impalements at 10–20 min of treatment.

In 17 single impalements, after a 1-min control, HCTZ effects were checked at a 10^{-4} M concentration of the drug. A small depolarization (+0.7 ± 0.1 mV, $P < 0.01$) already appeared during the first minute of treatment, but it was transient, disappearing spontaneously in a few minutes. Thus, the dose-response curve, which is difficult to obtain definitely, owing to the limited extent

[2] $$SE = \sqrt{\frac{\Sigma d_1^2 + \Sigma d_2^2}{n_1 + n_2 - 2}\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}$$

where d_1 and d_2 are the differences between the single results obtained under treatment or control conditions and the respective mean values and n_1 and n_2 are the numbers of observations of the respective data collectives.

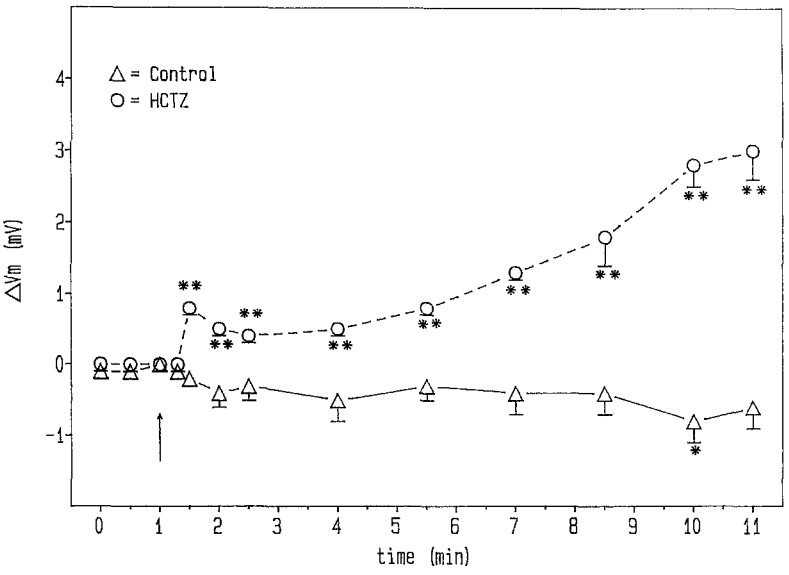

Fig. 1. Apical membrane potential variations (ΔV_m) under control conditions or on HCTZ treatment (single impalements). The 1-min V_m value has been taken as a reference. Results presented as means ± SE. The arrow shows HCTZ addition to the luminal medium. Control: n = 24; HCTZ (2.5×10^{-4} M): n = 9. *,**$P < 0.05$, $P < 0.01$ compared to the inner control.

of the depolarization, is probably between a $(0.8 - 1) \times 10^{-4}$ and $(2.5 - 5) \times 10^{-4}$ M dose.

HCTZ Added on the Serosal Side

The effects of HCTZ (2.5×10^{-4} M), added to the serosal medium for 60 min, were checked on V_m and V_{ms}, to observe possible basolateral actions or apical modifications on the cytoplasmic side. Both parameters did not change (*data not shown*); about 30 multiple impalements for the different data points).

Apical Conductance Changes Elicited by HCTZ

If HCTZ opens an apical Cl^- conductance, the reduction of Cl^- in the luminal saline should increase the depolarization described above.

The experiment involves some difficulties inasmuch as some secondary effects or counteractions might be elicited (Reuss, 1989). Yet, under control conditions it is generally accepted that no apical Cl^- conductance is revealed on this basis (*see above*). Actually, Table 2 shows that under control conditions, by lowering luminal Cl^- concentration from 125.3 to 30 mM during a single impalement, a V_m depolarization of only 3.6 mV is obtained, paralleled by a change in V_{ms} of 5.0 mV. These values are already corrected for the change in junction potential of the luminal 0.5 KCl-agar reference bridge, due to the luminal Cl^- lowering (for the correction procedure *see* Methods—Electrophysiological Measurements and[1]). The protocol and the time course of an uncorrected single impalement is reported in Fig. 2*a*. Since no significant change was observed by lowering Cl^- both for the transepithelial resistance (mean R_{ep} = 23.6 ± 2.4 Ωcm^2, n = 12) and for the apical/basolateral resistance ratio (mean R_m/R_s = 1.6 ± 0.1, n = 12), the formula reported by Cremaschi and Meyer (1982) was tentatively used to calculate the change in the apical electromotive force (ΔE_m), a cable analysis being impossible to carry out in this epithelium, due to its geometrical complexity. ΔE_m proved to be equal to 1.8 mV, a negligible value. However, when the epithelium was treated luminally with 2.5×10^{-4} M HCTZ for at least 10 min and impalements were then performed still under treatment, the luminal Cl^- lowering to 30 mM during an impalement elicited a significant depolarization of +6.2 mV (Table 2) paralleled by a ΔV_{ms} of 5.8 mV (data already corrected for the bridge junction potential whose artefact on the impalement is shown in Fig. 2*b*). The apical depolarization was significantly higher than the one measured under control conditions. A significant change was again not observed, in response to the Cl^- decrease, both for R_{ep} (mean: 20.9 ± 2.2 Ωcm^2, n = 8) and R_m/R_s (mean: 1.2 ± 0.2, n = 8), although R_m/R_s was in this case significantly lower ($P < 0.02$) than that measured in the absence of HCTZ and reported above (control conditions) as expected if HCTZ increases the apical conductance. Based on this, using the formula quoted above, a significant ΔE_m equal to +8.4 mV was calculated. Thus the result supports the opening of an apical Cl^- conductance by HCTZ, although the corrections and calculations, which must be applied to reach the conclusion, suggest caution.

Inhibitors of the Cl^- Conductance Elicited by HCTZ

Figure 3*a* shows the effects of some known inhibitors on the apical depolarization caused by HCTZ (analysis conducted with multiple impalements). DPC (10^{-4} M) in the lumen does not display any significant effect on the de-

Table 2. Effect of luminal Cl^- decrease from 125.3 to 30 mM on the apical membrane potential (V_m) and transepithelial p.d. (V_{ms}) under control conditions and HCTZ treatment (*a*). Calculation of the corresponding change in the apical electromotive force (ΔE_m)

Experimental condition	V_m (mV)	ΔV_m (mV)	V_{ms} (mV)	ΔV_{ms} (mV)	ΔE_m (mV)
Control					
Cl^- = 125.3 mM	−66.3 ± 1.7		−1.5 ± 0.3		
	(7)		(5)		
Cl^- = 30.0 mM	−62.7 ± 1.8*	+3.6 ± 1.0♦	+3.5 ± 0.3*	+5.0 ± 0.1♦	+1.8 ± 2.1
	(7)	(7)	(5)	(5)	(7)
HCTZ					
Cl^- = 125.3 mM	−64.0 ± 1.1		−2.0 ± 0.1		
	(10)		(5)		
Cl^- = 30.0 mM	−57.7 ± 1.6*	+6.2 ± 0.6♦*	+3.8 ± 0.5*	+5.8 ± 0.5♦	+8.4 ± 1.6
	(10)	(10)	(5)	(5)	(10)

(*a*) 2.5 × 10^{-4} M HCTZ in the lumen for at least 10 min before the impalement. During the impalement (prolonged for about 5 min) luminal Cl^- concentration was lowered for 2 min, then the high Cl^- saline was restored (HCTZ present for all the impalement period). Data presented as means ± SE (number of measurements in parentheses); results collected from 5 gallbladders.
* = $P < 0.01$ compared to the corresponding value obtained with Cl^- = 125.3 mM.
♦ = $P < 0.01$ compared to zero.
* = $P < 0.05$ compared to ΔV_m or ΔE_m under control conditions.

polarization which remains equal to 3–4 mV. Conversely, SCN^- (25 mM), furosemide (10^{-4} M), SITS (10^{-4} M) in the lumen abolish it. However, it is worth noting that SITS also converts the depolarization into a significant hyperpolarization of about −4 mV. Since at least SCN^- and SITS alone (i.e., not associated to HCTZ) do not modify the apical membrane potential (Fig. 4*b* and Cremaschi et al., 1987*b*), the overall result confirms that the HCTZ-dependent depolarization is due to the opening of a Cl^- conductance which seems to be SCN^-, furosemide and SITS sensitive.

Analysis of the Hyperpolarization

Under basal conditions no Cl^- conductance is present in the apical membrane (*shown above*) and SITS does not modify the apical membrane potential (Fig. 4*b* and Cremaschi et al., 1987*b*). Hence, the hyperpolarization observed on HCTZ-SITS treatment cannot be ascribed to the closure by SITS of a basal Cl^- conductance parallel to the one elicited by HCTZ. Based on the hyperpolarizing K^+ conductance opened by HCTZ in the fish urinary bladder (Duffey & Frizzell, 1984), we assumed that, in gallbladder, HCTZ concomitantly opens a Cl^- and a K^+ conductance, with opposite effects on the apical membrane potential. Then, based on the considerations reported in the Discussion, we started to use apamin (10^{-8} M in the lumen) as a possible inhibitor of the K^+ conductance. The analysis was performed with sets of multiple impalements. Figure 3*b* shows that the hyperpolarization is abolished by apamin. On the other hand,

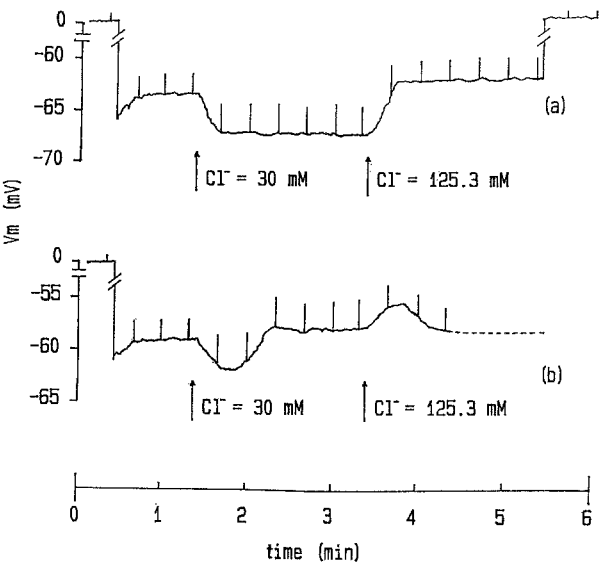

Fig. 2. Record of tracings obtained from impalements taken under control conditions (*a*) or after a HCTZ treatment of at least 10 min (*b*), during which luminal Cl^- concentration was lowered to 30 mM. Tracings are not corrected for the junction potential change of the luminal 0.5 M KCl-agar reference bridge caused by the Cl^- lowering; in the same way the voltage deflections elicited by current pulses include drops on the luminal extracellular saline. The increase in deflection, when luminal Cl^- is 30 mM, is only due to the changes in these extracellular drops. Trace (*b*), obtained under HCTZ treatment, is interrupted (broken line) after the return to the high Cl^- saline, the record of the artifact imposed by junction potential change at the bridge boundary and a nearly complete recovery of V_m. At this point impalements were generally lost, probably because of cell volume changes. ↑ luminal saline change.

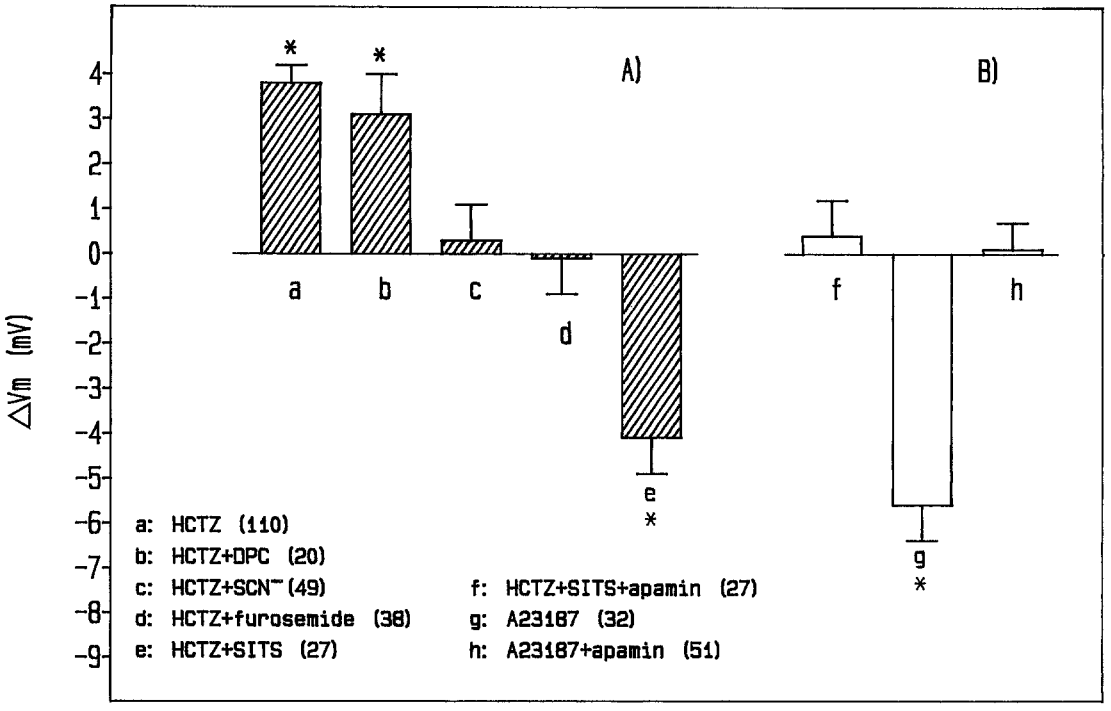

Fig. 3. (*A*) Effects of DPC, SCN^- furosemide and SITS on the apical depolarization elicited by HCTZ. (*B*) Effects of apamin on the hyperpolarization elicited by (HCTZ + SITS) or A23187. Multiple impalements. Bars represent means ± SE (number of impalements in parentheses). Number of gallbladders: 15, 2, 6, 4, 5 respectively for *a, b, c, d-e-f-g, h* conditions. HCTZ: 2.5×10^{-4} M; DPC, furosemide, SITS: 10^{-4} M; SCN^-: 25×10^{-3} M; apamin: 10^{-8} M; A23187: 10^{-5} M. Treatments on the luminal side only. *: $P < 0.01$ compared to zero.

this inhibitor has no effect under basal conditions (Fig. 4*b*).

Since apamin is a specific inhibitor of a class of Ca^{2+}-sensitive K^+ channels, a countercheck of the presence of these channels in the membrane and of their possible opening was obtained with the ionophore A23187 (10^{-5} M in the lumen) which introduces Ca^{2+} into the cell: a significant hyperpolarization of –5.6 mV was observed which was abolished by 10^{-8} M apamin (Fig. 3*b*). The hyperpolarization was significantly smaller with a 0.5×10^{-5} M concentration of the ionophore (*data not shown*).

Ca^{2+}-INSENSITIVE Cl^- CHANNELS

On this basis, the actual complete depolarization elicited by HCTZ should be measured in the presence of apamin (10^{-8} M in the lumen). Under these conditions (HCTZ-apamin), the depolarization increases significantly ($P < 0.01$) from 3.8 to 7.2 mV (Fig. 4*a*). Similar increasing effects of the depolarization are displayed by 10^{-4} M nifedipine (inhibitor of Ca^{2+} channels and of Ca^{2+}-activated K^+ channels) and 10^{-5}–10^{-4} M verapamil (putative inhibitor of Ca^{2+} channels, *see* Discussion and [3]). Thus HCTZ only indirectly seems to open the apamin-sensitive, Ca^{2+}-activated K^+ channels, probably directly opening verapamil-sensitive Ca^{2+} channels and introducing Ca^{2+} into the cell (*see* Discussion and [3]). If this entry is hindered by verapamil the depolarization is preserved and developed at its maximal extent. Hence the Cl^- channels opened by HCTZ seem to be Ca^{2+} insensitive.

Nifepidine and verapamil as well as apamin have no effect under basal conditions (Fig. 4*b*); thereby under these conditions no Ca^{2+}-activated K^+ channels seem to be open.

[3] Verapamil is considered a selective blocker of Ca^{2+} channels at 10^{-5} M concentration (Hagiwara & Bierly, 1981), at which we obtained already maximal effects. Ca^{2+} antagonists also interfere with Na^+ and K^+ channels (Hagiwara & Bierly, 1981; McDonald et al., 1994). Among K^+_{Ca} channels, some of them (apamin insensitive, 18 pS, erythrocytes) are reported to be inhibited by dihydropyridines (concentrations much higher than 10^{-5} M for maximal effects being reached); yet they are not inhibited by verapamil even at 10^{-4} M concentration (Kaji, 1990). Conversely, many Ca^{2+}-insensitive K^+ channels are inhibited by verapamil and its derivatives, but maximal effects are again reached, in any case, at concentrations much higher than 10^{-5} M (Hume, 1985; Hillyard & Van Driessche, 1992). Although these observations on one side support the involvement of Ca^{2+} channels in the HCTZ effects on gallbladder epithelium, on the other side they suggest some caution for a definitive conclusion.

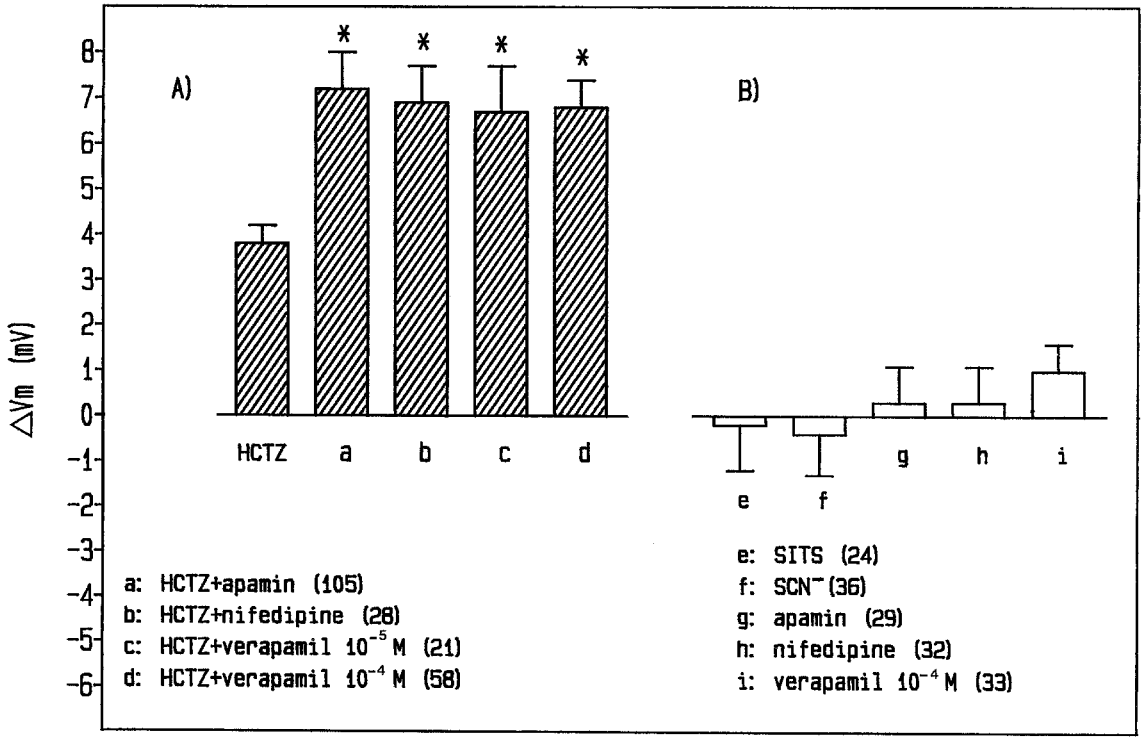

Fig. 4. (*A*) Effects of apamin or nifedipine or verapamil on the depolarization caused by HCTZ. (*B*) Insensitivity of the apical membrane potential (V_m) to SITS, SCN^-, apamin, nifedipine, verapamil under basal conditions. Multiple impalements. Bars represent means ± SE (number of impalements in parentheses). Number of gallbladders: 10, 6, 4 respectively for *a, d, b-c-e-f-g-h-i* conditions. HCTZ: 2.5×10^{-4} M; apamin: 10^{-8} M; nifedipine 10^{-4} M; verapamil: 10^{-5} and 10^{-4} M; SITS: 10^{-4} M; SCN^-: 25×10^{-3} M. Treatments on the luminal side only. The bar concerning HCTZ is taken from Fig. 3 as a reference. *:$P < 0.01$ compared to zero.

Single Impalements

Apical membrane potential changes elicited by different treatments were also checked by maintaining the microelectrode in the same cell for 21 min after the stability was reached. Figures 5 and 6 report directly the mean apical membrane potential variations over time obtained from many of such impalements. If after a 1-min control, the apical membrane was simultaneously treated with 2.5×10^{-4} M HCTZ and 10^{-4} M SITS, the closure of Cl^- channels with SITS not only abolished the typical depolarization due to HCTZ, but also evidenced a hyperpolarization which, already significant in 30 sec, developed gradually and reached its maximum (about 4 mV) in 9 min (Fig. 5). It was completely reversible (*not shown*). This hyperpolarization was abolished by 10^{-8} M apamin (Fig. 5), whereas apamin alone did not change the apical membrane potential (Fig. 6), all this confirming that Ca^{2+}-sensitive K^+ channels, closed under control conditions, are opened by HCTZ treatment. In the presence of apamin or 10^{-4} M verapamil, Ca^{2+}-activated K^+ channels being maintained closed, the depolarization evoked by HCTZ developed at its maximal extent (about 7 mV) (Fig. 6). It is worth noting that the maximum depolarization was revealed as equal with apamin or verapamil and that the maximum was reached in 7.5–9 min.

Parameters Measured to Evaluate the Cl^- Conductance

With the aim of estimating the Cl^- conductance opened by HCTZ, we performed measurements of the apical and the transepithelial potential difference, of the apical and basolateral resistance ratio and the transepithelial resistance under control conditions and on treatment with HCTZ-verapamil (to obtain a change of the apical conductance caused only by the opening of Cl^- channels). The protocol was the usual for multiple impalements. Results are reported in Table 3: V_m is largely depolarized (as expected from what is reported in Fig. 4*a*), V_{ms} and R_m/R_s decrease significantly, R_{ep} is statistically unmodified. It is worth noting that R_m/R_s decreases due to an increase of ΔV_s without a significant change in ΔV_m. This is in agreement with an increase in the electrical current crossing the cell, elicited by a reduced R_m. Moreover, it should be emphasized that the decrease in V_{ms} is significant but small (about 0.6 mV), a value compatible

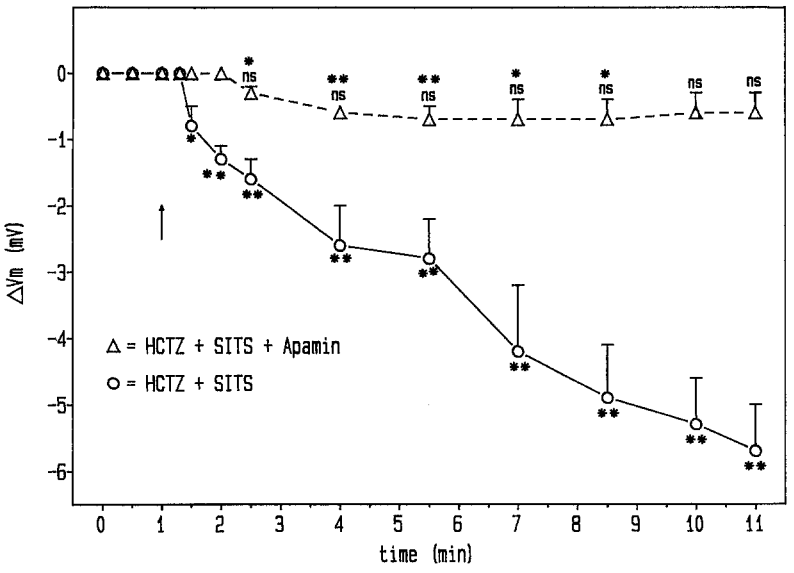

Fig. 5. Apical membrane potential variation (ΔV_m) on HCTZ + SITS or HCTZ + SITS + apamin treatment (single impalements). The 1-min Vm value has been taken as a reference. Results presented as means ± SE. The arrow shows the addition of the drugs to the luminal medium. HCTZ: 2.5×10^{-4} M; SITS: 10^{-4} M; apamin: 10^{-8} *M*. HCTZ + SITS experiments: n = 10; HCTZ + SITS + apamin experiments: n = 5; *,**:$P < 0.05$ or $P < 0.01$ compared to the inner control; NS not significant compared to the external control reported in Fig. 1.

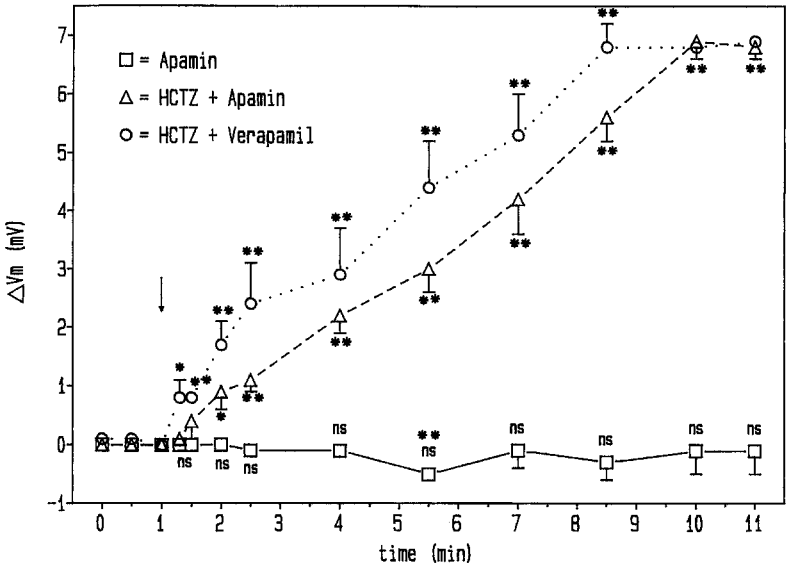

Fig. 6. Apical membrane potential variations (ΔV_{mn}) on apamin or HCTZ + apamin or HCTZ + verapamil treatment (single impalements). The 1-min V_m value has been taken as a reference. Results presented as means ± SE. The arrow shows the addition of the drugs to the luminal medium. HCTZ: 2.5×10^{-4} M; apamin: 10^{-8} M; verapamil: 10^{-4} M. Apamin experiments: n = 5; HCTZ + apamin experiments: n = 4, HCTZ + verapamil experiments: n = 4; *,**$P < 0.05$ or $P < 0.01$ compared to the inner control; NS not significant compared to the external control reported in Fig. 1.

with the merely cellular action of HCTZ and with the entity of the paracellular short-circuit effect. Accordingly, it has previously been shown that HCTZ does not modify the paracellular ionic selectivity (Cremaschi & Porta, 1994). Finally, it should be stressed that in this animal group the V_m depolarization (+10.6 mV) is greater than that reported in Fig. 4*a* for similar experiments (about +7 mV). Thus the Cl^- conductance, which is possible to estimate on this basis, represents its top limit.

Discussion

In the urinary bladder of the winter flounder, HCTZ inhibits Na^+-Cl^- symport and concomitantly hyperpolarizes the apical membrane potential by increasing the apical K^+ conductance (Duffey & Frizzell, 1984; Stokes, 1984; Ziyadeh, Kelepouris & Agus, 1987). In the rabbit gallbladder, the symport inhibition is unexpectedly paralleled by the generation of an apical depolarization (Cremaschi et al., 1992).

In this epithelium, under control conditions, the apical membrane does not display any Cl^- conductance (*see* Introduction) and the apical membrane potential is not affected either by the Cl^- lowering in the lumen (Hénin & Cremaschi, 1975; *present paper*) or by some putative inhibitors of Cl^- channels such as SCN^- and SITS (Cremaschi et al., 1987*b; present paper*). In accordance with the inhibition of the transepithelial NaCl transport the depolarization elicited by HCTZ might be accounted for

Table 3. Parameters measured to evaluate the Cl^- conductance elicited by HCTZ (multiple impalements)

Experimental condition	V_m (mV)	V_{ms} (mV)	R_m/R_s	R_{ep} ($\Omega \cdot cm^2$)
Control	-65.2 ± 1.3 (11)	-1.0 ± 0.2 (11)	1.5 ± 0.1 (11)	23.7 ± 1.2 (11)
HCTZ + Verapamil	-55.8 ± 0.8* (13)	-0.4 ± 0.1* (13)	0.7 ± 0.1* (13)	21.3 ± 1.9 (13)

Results presented as means ± SE with the number of measurements in parenthesis. The transepithelial electric potential difference (V_{ms}), the apical/basolateral resistance ratio (R_m/R_s), the transepithelial resistance (R_{ep}) were determined each time an apical potential difference (V_m) measurement was obtained, so that the reported values are all strictly correlated.
* = $P < 0.01$.

by the opening of an apical Cl^- conductance allowing Cl^- to escape back to the lumen from the cell, where it has been accumulated by symport above the value predicted for electrochemical equilibrium.

In fact, with HCTZ treatment, a SITS inhibitable increase in Cl^- backflux was observed radiochemically (Cremaschi & Porta, 1994). Moreover, this paper shows that the apical membrane potential becomes sensitive to the luminal Cl^- concentration, the depolarization elicited by HCTZ being significantly enhanced by the Cl^- lowering in the lumen. Furthermore, depolarization is apparently abolished by SCN^-, furosemide and SITS.

The latter inhibitor, which has no effect under control conditions, also converts the depolarization into hyperpolarization. This suggests that in gallbladder two different conductances, generating opposite effects and partially masking each other, are opened by HCTZ; the first depolarizing, the second hyperpolarizing the membrane as well as in the urinary bladder. In the gallbladder SCN^- and furosemide, at the concentration used, would only be able to inhibit partially the depolarizing Cl^- conductance, whereas SITS would abolish it, fully revealing the effects of the hyperpolarizing conductance.

In agreement with the results obtained in the winter flounder urinary bladder (Duffey & Frizzell, 1984) we supposed that the hyperpolarizing conductance was selective to K^+.

Moreover, in the urinary bladder, Ziyadeh, Kelepouris & Agus (1987) found that thiazides stimulate calcium absorption concomitantly with the generation of the apical hyperpolarization. They assumed that the decrease in intracellular Na^+ activity, related to the inhibition by thiazides of the apical Na^+-Cl^- symport, can enhance a putative basolateral Na^+/Ca^{2+} exchange and hence the transepithelial Ca^{2+} absorption. Similar findings and interpretations have been reported for the distal tubule (*see* e.g., Costanzo & Windhager, 1978; Suki, 1979; Gesek & Friedman, 1992). In the latter tissue ^{3}H-metolazone (a diuretic drug similar to thiazides) has been found to bind to high affinity sites (corresponding to the thiazide-sensitive Na^+-Cl^- symport) and low affinity sites from which metolazone is displaced not only by the thiazides themselves but also, and with higher affinity, by compounds from the three major classes of calcium antagonist drugs, that is dihydropyridine, phenilalkylamine and benzothiazepine (Beaumont, Vaughn & Fanestil, 1988).

On these grounds, let us suppose that the low affinity sites are related to calcium channels and that these channels can be opened by metolazone and thiazides (and of course closed by calcium antagonists). Hence, it can be suggested that the transepithelial transport of calcium in the urinary bladder and distal tubule can be enhanced by thiazides not only because of the decrease in intracellular Na^+ and the consequent increase in the basolateral Na^+/Ca^{2+} exchange, but also because of an enhanced apical Ca^{2+} entry; moreover, it can be assumed that the hyperpolarizing conductance, elicited by HCTZ, is related to Ca^{2+} sensitive K^+ channels.

Therefore, we started to use apamin, at a 10^{-8} M concentration, as a highly selective drug inhibiting a class of these channels (SK channels: Blatz & Magleby, 1986). The results clearly show that apamin is ineffective under basal conditions but abolishes the hyperpolarization elicited by HCTZ. Moreover, by leaving Cl^- channels open (absence of SITS treatment), the K^+ channel block by apamin reveals the entire depolarization elicited by HCTZ. Equal depolarization is evidenced in the same conditions with verapamil (a putative blocker of Ca^{2+} channels: Hagiwara & Byerly, 1981) or with nifedipine (a blocker of Ca^{2+} channels at nanomolar concentrations, but also of Ca^{2+} activated K^+ channels at the concentration used; *see* e.g., Kaji, 1990). Further indication of the presence of apamin-sensitive, Ca^{2+}-activated K^+ channels in this membrane is given by the abolition by apamin of the hyperpolarization caused by A23187 ionophore.

Altogether these results demonstrate that HCTZ raises intracellular Ca^{2+} activity (probably opening apical, verapamil-sensitive Ca^{2+}-channels[3] and, as a consequence, opens apamin-inhibitable, Ca^{2+}-sensitive K^+ channels (SK channels). The process is relatively slow as it requires subsequent steps.

SK channels have low conductance (6–14 pS), are nearly insensitive to external tetraethylammonium (TEA), are unaffected by charybdotoxin (CTX) and very

slightly affected by voltage so that they can be active at all membrane potentials (−90 to +40 mV) with 10^{-5}–10^{-6} M intracellular Ca^{2+}; however, they are much more Ca^{2+} sensitive (and thus open) at negative membrane potentials (Blatz & Magleby, 1986; Lang & Ritchie, 1990). They have been identified in several tissues and cells (smooth and skeletal muscle, some neurons, GH_3 pituitary cells, adrenal cortex, hepatocytes and enterocytes).

By far the most important apical conductance in gallbladder is that of the K^+ in the rabbit, guinea-pig, *Necturus* and toad and part of this conductance, in *Necturus,* has been shown to be voltage dependent, TEA inhibitable and sensitive to intracellular Ca^{2+} (for a review *see* Cremaschi & Porta, 1992). By using the patch clamp technique combined with intracellular microelectrode studies it has been shown that these properties are related to the existence in the apical membrane of *Necturus* of TEA-inhibitable, Ca^{2+}-activated maxi K^+ channels, with conductance of about 200 pS, which account for roughly 15% of the apical membrane conductance at rest; their importance increases when the membrane is depolarized and intracellular Ca^{2+} increased (Segal & Reuss, 1990*a, b*). Similar maxi K^+ channels (BK channels) have also been observed in gallbladders of other species, such as *Triturus pyrrhogaster* (Maruyama, Matsumaya & Hoshi, 1986) and guinea-pig (Meyer, Cremaschi & Bottà, 1992), and are widespread in excitable and epithelial cells (*see* Segal & Reuss, 1990*a, b*) where they prove to be insensitive to apamin (Blatz & Magleby, 1986), but blocked by charybdotoxin (Miller et al., 1985).

Thereby, the existence of apamin-sensitive, Ca^{2+}-activated K^+ channels in rabbit gallbladder is a new indication for gallbladder and deserves some comments. In the rabbit they are alternative to maxi K^+ channels in regulating the apical membrane potential or they are present together with maxi K^+ channels as it occurs for example in the rat skeletal muscle and GH_3 pituitary cells (Blatz & Magleby, 1986; Lang & Ritchie, 1990). Since BK channels, at the negative membrane potentials measured at rest, are much less sensitive to Ca^{2+} than SK channels (Blatz & Magleby, 1986), it is possible that the increase in intracellular Ca^{2+} activity elicited by HCTZ or A23187 (at the luminal concentration used) is sufficient to activate substantially SK channels but is too low for BK channels. This can explain why the entire hyperpolarization observed is apamin sensitive.

The presence of hyperpolarizing K^+ channels, with regulatory importance, on the apical membrane, across which essentially neutral transports occur, can be perhaps explained by the consequent reflected hyperpolarization which appears on the basolateral membrane, owing to the shunting effect of the leaky paracellular pathway. This effect may be important in maintaining a high basolateral electrochemical potential gradient when across the basolateral membrane the Cl^- conductance increases, to sustain an increased apical neutral transport of NaCl, as it occurs e.g., when bicarbonate is present in the incubating media (Cremaschi et al., 1993; Stoddard & Reuss, 1988).

Elicited Cl^- Conductance

The apical depolarization elicited by HCTZ starts to appear 20–30 sec after the beginning of the treatment. A delay of some 10 sec is expected, based on the renewal time of the saline in the luminal chamber and the attainment of the HCTZ final concentration in the apical unstirred layer (Cremaschi et al., 1987*b*). Thus the HCTZ effect on Cl^- conductance starts very early; yet, it is progressive and takes 7.5–9 min to be accomplished. Therefore, either the drug operates on the cytoplasmic side of the apical membrane and the effect increases with the progressive entry of the drug into the cell or HCTZ acts on the external side but, after binding to the membrane, its target is only slowly modified by the interaction. The fact that HCTZ is ineffective on the serosal side seems to rule out a possible action on the inner side of the apical membrane.

The elicited Cl^- conductance is inhibited by SITS, furosemide and SCN^- (although the latter two drugs are unable to abolish it, at least at the concentration used), but is insensitive to DPC. The SCN^- counteraction may be explained not only by an inhibition but also by a SCN^- electrodiffusion through the Cl^- pathway from the outside to the inside the cell and, thus in contrast with the Cl^- effect. Actually, conductive Cl^- pathways are frequently permeable to SCN^- (e.g., Copello et al., 1993).

Cl^- conductance seems not to be indirectly elicited by HCTZ by the increase of intracellular Ca^{2+} concentration as it does not open, also in the absence of HCTZ, on treatment with A23187 ionophore. Conversely, it remains open, on treatment with HCTZ, also in the presence of verapamil, when intracellular Ca^{2+} activity probably does not increase. The absence of Ca^{2+} sensitive Cl^- conductance is in accordance with what is found in the apical membrane of *Necturus* gallbladder epithelium, where Ca^{2+}-activated Cl^- channels are not detected (Heming, Copello & Reuss, 1994). Based on all this, it is impossible to support the unifying hypothesis that HCTZ only acts to enhance intracellular Ca^{2+}, thus opening both K^+ and Cl^- channels. By contrast, the conclusion emerging is that HCTZ probably acts directly on Ca^{2+} channel, consequently on K^+ channels and independently on Cl^- conductance.

It has been shown that some thiazides can inhibit the phosphodiesterase and enhance the intracellular level of cyclic AMP (Moore, 1968; Vulliemoz, Verosky & Triner, 1980). Cl^- conductance opening might then be mediated by cAMP. However, the effect on phosphodiesterase was obtained at very high doses. Moreover, al-

though cAMP opens Cl^- channels in *Necturus* and guinea-pig gallbladders where it leads to secretion into the lumen (Petersen & Reuss, 1983; Stewart et al., 1989), there is no evidence for such an action in the rabbit where it only inhibits or partially inhibits absorption (Mertens, Wheeler & Mayer, 1974; Frizzell, Dugas & Schultz, 1975). Finally, the Cl^- conductance elicited by cAMP in *Necturus* gallbladder is insensitive to SITS and furosemide (Copello et al., 1993); thereby, it has pharmacological properties different from those of the Cl^- conductance elicited by HCTZ.

An attractive hypothesis is that the HCTZ-dependent Cl^- conductance represents merely an increase of the inner basal Cl^- conductance present in the Cl^-/HCO_3^- exchange (Kanuf, 1979; Rothstein & Ramjeesingh, 1980). In favor of this it can be emphasized that: (i) the exchanger is present in rabbit gallbladder even if it is functionally silent under the experimental conditions used here (Cremaschi et al., 1983, 1987*a*), (ii) in fish urinary bladder, where Cl^-/HCO_3^- exchange is absent (Stokes, 1984), HCTZ does not elicit any Cl^- conductance, (iii) the elicited conductance is SCN^-, furosemide and SITS sensitive, like the exchanger and its basal conductive pathway (Knauf, 1979; Rothstein & Ramjeesingh, 1980; Cremaschi et al., 1983), (iv) HCTZ has been shown to inhibit the exchanger (Cousin, Motais & Sola, 1975; Ferriola, Acara & Duffey, 1986; Karnisky & Aronson, 1987; Porta & Cremaschi, 1993). Apparently, against this hypothesis is the fact that DPC, which is able to inhibit the exchanger (Reuss, Costantin & Bazile, 1987), has no effect on conductance. However, there is no evidence that this drug is also able to inhibit the inner basal conductance of the exchanger; moreover, HCTZ itself inhibits the exchanger and possibly even increases its inner conductance.

The HCTZ-dependent Cl^- conductance cannot be measured by cable analysis due to the complex geometry of the rabbit gallbladder surface. However, it can be estimated by measuring apical membrane and transepithelial p.d., the apical/basolateral resistance ratio and transepithelial resistance both under control or treatment (HCTZ-verapamil) conditions. Let us suppose that HCTZ modifies only R_m and E_m. The paracellular pathway does not change its selectivity (Cremaschi & Porta, 1994) and the transepithelial resistance is not significantly modified; moreover, the drug does not act on the basolateral side and this probably also rules out its entrance into the cell. Based on this and on the equations derived by Hénin et al (1977) for a similar condition, it is possible to calculate the paracellular, the basolateral and the apical resistance (Table 4). The latter is found to decrease considerably under HCTZ-verapamil treatment. From the difference of the corresponding apical conductances under control and treatment conditions (G_m, G'_m), the elicited Cl^- conductance (G_{Cl}) is calculated, Ca^{2+} and K^+ conductances being maintained closed by verapamil

Table 4. Evaluation of the Cl^- conductance elicited by HCTZ

Used parameters*		Calculated parameters	
ΔV_m (mV)	+9.4	R'_{sh} ($\Omega \cdot cm^2$)	23.4
ΔV_{ms} (mV)	+0.6	R_s ($\Omega \cdot cm^2$)	330
R_m/R_s	1.5	R_m ($\Omega \cdot cm^2$)	495
R'_m/R_s	0.7	R'_m ($\Omega \cdot cm^2$)	231
R_{ep} ($\Omega \cdot cm^2$)	22.5	G_m ($S \cdot cm^{-2}$)	2×10^{-3}
		G'_m ($S \cdot cm^{-2}$)	4.3×10^{-3}
		G'_{Cl} ($S \cdot cm^{-2}$)	2.3×10^{-3}
		t'_{Cl} (G'_{Cl}/G'_m)	0.53

* Taken from Table 3. R_{ep} is the mean of the transepithelial resistance measured under control or treatment conditions. ΔV_m and ΔV_{ms} are the difference of the respective V_m and V_{ms} measured under treatment and control conditions. R'_m/R_s, R'_{sh}, R'_m, G'_m, G'_{Cl}, t'_{Cl}: parameters measured or calculated under treatment conditions; t'_{Cl}: transport number under treatment conditions.

treatment. From the G'_{Cl}/G'_m ratio a transport number $t'_{Cl} = 0.53$ is derived which shows that G'_{Cl} under HCTZ treatment is largely increased from zero to a value similar to that of the basal K^+ conductance.

Based on G'_{Cl} calculated in this way, on V_m and intracellular Cl^- activity measured at the same time (15 min) under 10^{-3} M HCTZ treatment, a I_{Cl} (cell to lumen) equal to 52.0 $\mu A\ cm^{-2}$ and an equivalent cell-to-lumen backflux (J_{Cl}) equal to 1.94 $\mu mol\ cm^{-2}\ h^{-1}$ can be determined. This value compares with a value of about 10 $\mu mol\ cm^{-2}\ h^{-1}$ of influx of the Na^+-Cl^- symport concomitantly inhibited by HCTZ and an equivalent value of about 10 $\mu mol\ cm^{-2}\ h^{-1}$ of influx homeostatically elicited and supported by the Na^+-K^+-$2Cl^-$ symport (Cremaschi et al., 1992). Since under these conditions the intracellular Cl^- accumulation decreases by a maximum of 60–70%, the Cl^- backflux contributes substantially to its dissipation. It should be emphasized that the electrodiffusive backflux value calculated here is similar to that which can be inferred approximately from radiochemical measurements (Cremaschi & Porta, 1994).

It is worth noting that the dose-response relationship is different for the action of HCTZ on the transepithelial NaCl transport and the Cl^- conductance. 10^{-4} M HCTZ only minimally and transiently affects V_m, although it produces a significant decrease (about 30%) in the transport; 2.5×10^{-4} M HCTZ displays maximal, or nearly maximal effects on V_m (and G_{Cl}), whereas it reduces the transport by only less than 60%. All this is in agreement with a dual action on the apical membrane (opening of the Cl^- conductance, inhibition of the symport).

Finally, as for possible physiological implications of the Cl^- conductance opening by HCTZ, at present the obtained results simply better characterize the pharmacological action of HCTZ on the transepithelial transport of NaCl, without particularly evident physiological involvements. At most this may indicate the presence of a regulatory Cl^- channel at the apical membrane of gall-

bladder epithelium, provided that the Cl^- conductance elicited by HCTZ does not merely represent an increase in the intrinsic conductance of the Cl^-/HCO_3^- exchanger as, by contrast, we are inclined to think. In the latter case HCTZ can be an important tool to better study the exchanger besides NaCl symport.

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