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Intracellular Chloride Activity and Apical Membrane Chloride Conductance in *Necturus* Gallbladder

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Summary. Open-tip and Cl^--selective microelectrodes were used to study the effect of external pH on apical membrane potential (V_a) and intracellular chloride activity (a^i_{Cl}) in epithelial cells of *Necturus* gallbladder. Increasing the pH from 7.2 to 8.2 in the mucosal, the serosal, or in both bathing solutions simultaneously, hyperpolarized V_a (control value -60 ± 5 mV) by about -6, -10 and -17 mV, respectively, but did not significantly change the transepithelial potential ($V_T=0.3\pm0.5$ mV). Identical hyperpolarizations were recorded with Cl^--selective microelectrodes, even 40 min after changing external pH. Thus, a^i_{Cl} (12 ± 2 mM) remained essentially constant. The ratio fV_a between the deflections in V_a and V_T produced by transepithelial current pulses, which is an approximate measure of the fractional resistance of the apical membrane, decreased when mucosal pH was increased, and increased when serosal pH was raised. The changes in V_a and fV_a are due, in part at least, to the known pH dependence of cell membrane K^+ conductance (P_K) in this tissue. The constancy of a^i_{Cl}, despite significant increases in V_a, indicates that cell membrane Cl^- conductance (P_{Cl}) is virtually zero or decreases, with increased external pH, in a way that compensates for the increased driving force for Cl^- exit. Experiments in which 90 mM gluconate or 90 mM methylsulfate were substituted for an equivalent amount of luminal Cl^- did not provide any evidence for a significant contribution of Cl^- ions, per se, to the emf or conductance of the apical membrane. They suggested, rather, a dependence of apical membrane cation permeability on luminal Cl^- concentration. Since basolateral membrane P_{Cl} is known to be very low, the insensitivity of a^i_{Cl} to V_a is the consequence of a negligible elctrodiffusive Cl^- permeability at both cell membranes. Thus, overall, transcellular Cl^- transport in *Necturus* gallbladder is, in large measure, effected by electroneutral processes.

Key Words pH, membrane potentials · intracellular chloride · *Necturus* gallbladder · chloride activity · chloride conductance

Introduction

Chloride entry into the epithelial cells of the gallbladder seems to be mediated by an electroneutral coupled NaCl transport mechanism located in the apical cell membrane (Frizzell, Field & Schultz, 1979; Reuss & Grady, 1979; Frizzell & Duffey, 1980; Garcia-Diaz & Armstrong, 1980). There has been, and still is, some uncertainty whether and to what extent neutral NaCl influx is attributable to a simple symport of Na^+ and Cl^-, or to the operation of parallel, linked exchange (antiport) processes, i.e. Na^+/H^+ and Cl^-/OH^- (HCO_3^-) exchanges. Recent evidence (Ericson & Spring, 1982; Weinman & Reuss, 1982) indicates that, in *Necturus* gallbladder, NaCl symport is the major mechanism responsible for steady-state transepithelial salt and water absorption. Na^+/H^+ and Cl^-/OH^- exchange on the other hand appear to be mainly implicated in cellular homeostasis, e.g. volume regulation in response to hyperosmotic shock (Ericson & Spring, 1982). In any event the energy for Cl^- entry is ultimately derived from the Na^+ gradient across the apical membrane. This, in turn, is maintained by the basolateral Na^+ pump. Thus, it is not surprising that luminal Na^+ concentration has an important effect on intracellular Cl^-. When luminal Na^+ concentration is at the normal level, the intracellular Cl^- activity, a^i_{Cl}, exceeds the value consistent with the assumption that Cl^- is in electrochemical equilibrium across the apical cell membrane. Removal of luminal Na^+ causes a^i_{Cl} to decline to a level close to the calculated equilibrium value.

Other factors affecting a^i_{Cl} may give clues to the mechanisms responsible for the exit of Cl^- from the cell. The exact nature of these mechanisms is not yet clear. In *Necturus* gallbladder, the Cl^- conductance of the basolateral cell membrane is much too small to account for observed rates of Cl^- transport (van Os & Slegers, 1975; Reuss, 1979). Therefore some kind of electroneutral process or processes must be invoked as the major mechanism of Cl^- movement across this membrane. Similar conclusions apply to the basolateral

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cell membrane of *Necturus* proximal tubule (Shindo & Spring, 1981; Guggino, Boulpaep & Giebisch, 1982).

In the experiments reported here we investigated the effects of changes in pH, in the physiological range, on the apical membrane potential V_a, and on a^i_{Cl} in *Necturus* gallbladder. We show that, despite significant changes in V_a, a^i_{Cl} remained constant following alterations of external pH. In addition, replacement of luminal Cl^- by gluconate or methylsulfate did not provide evidence for a significant electrodiffusive Cl^- movement across the apical cell membrane. Thus, transcellular Cl^- transport in *Necturus* gallbladder appears, to a large extent, to be mediated by electroneutral processes at both the apical and basolateral cell membranes.[1]

Materials and Methods

Necturus maculosus obtained from Graska Biological Supplies (Oshkosh, WI) were kept in a large aquarium at 4° C. The animals were killed by a blow on the head and a double transection of the spinal cord was performed. The abdominal cavity was opened by median incision, the gallbladder was removed, cleaned from adhering liver tissue, emptied, and cut longitudinally. It was then washed free of bile residues in Ringer's solution and mounted (mucosal surface upward) as a flat sheet at room temperature (23 ± 1 °C) in a divided chamber. Both the luminal and serosal surfaces of the tissue were independently and continuously perfused by gravity. To ensure homogeneous distribution over the entire luminal surface, the perfusion fluid entered the luminal compartment through six fine channels above the surface of the epithelium. This, combined with the small volume (0.2 ml) of the luminal compartment and a fast perfusion rate, allowed complete exchange of the luminal bathing fluid in less than 10 sec. The effective exposed area of gallbladder was 0.38 cm^2. Stopcock silicone grease (Dow Corning) was used to prevent edge damage and leakage. The serosal surface was supported by a stainless steel grid. A negative hydrostatic pressure of approximately 20 cm H_2O was applied to the serosal compartment both to attach the bladder to the supporting grid and to drive the serosal perfusion. Due to the dead spaces in the tubing and the serosal half-chamber, and to the unstirred layers of the grid and connective tissue, changes in electrical parameters due to alterations in the serosal fluid were observed only after a delay period of 50 to 60 sec. Both perfusion solutions could be rapidly changed either by manual stopcocks or by an electrically activated solenoid valve (Model 330, Angar Scientific) located near the chamber inlets.

The Ringer's solutions contained, in mM: 100.0 NaCl, 2.5 KCl, 1.0 $CaCl_2$ and were buffered to pH 7.2 or 8.2 with 5 mM imidazole-Cl or Tris-Cl, respectively. In experiments where luminal Cl was reduced, 90 mM NaCl were replaced by corresponding amounts of Na gluconate or Na methylsulfate. Both perfusion solutions were continuously bubbled with 100% O_2.

[1] Preliminary accounts of some of these experiments have been given elsewhere (Corcia, Garcia-Diaz, & Armstrong, 1982; Garcia-Diaz & Armstrong, 1982).

Single-barreled open-tip and Cl^--selective microelectrodes were made as described by Garcia-Diaz and Armstrong (1980). Briefly, micropipettes were drawn from "Kwik-Fil" borosilicate glass capillary tubing (1.2 mm o.d., W.P. Instruments) in a vertical puller (Model 700 C, D. Kopf Instruments). Open-tip microelectrodes were filled with 3 M KCl and had resistances between 8 and 40 MΩ when immersed in Ringer's solution. More stable impalements were obtained with microelectrodes having resistances in the range 10 to 20 MΩ. Micropipettes for Cl^--selective microelectrodes were silanized with a solution of 0.4% polymethylhydrogen siloxane (1107 fluid, Dow Corning) in acetone. The liquid Cl^- exchanger used was Corning 477315 (Corning Medical). Calibration of these microelectrodes in solutions of 10, 20, 50 and 100 mM KCl gave slopes S that ranged from 52 to 57 mV/decade change in Cl^- activity (mean 54.7). The selectivity ratio against HCO_3^-, $K^{-1}_{Cl-HCO_3}$, measured by the separate solution method in 0.1 M Na-anion solutions, ranged from 7.6 to 12.5.

Intracellular Cl^- activity was calculated from the equation

$$a^i_{Cl} = a^o_{Cl}\, 10^{(V_a - V_{Cl})/S}$$

where V_a is the mean apical membrane potential measured in the same tissue under the same conditions with a conventional open-tip microelectrode, V_{Cl} is the change in the potential recorded with the Cl^--selective microelectrode upon impalement, S is the slope of the microelectrode measured during calibration and a^o_{Cl} is the external Cl^- activity (approximately 83 mM).

Electrical Measurements

The transepithelial potential difference V_T was continuously measured by two calomel half-cells connected to the mucosal and serosal solutions by either 3 M KCl-agar or Ringer's-agar bridges (*see below*). The tissue was kept in the open-circuit mode except for brief periods when transepithelial current (I_T) pulses were applied via two AgCl-coated silver rings. Microelectrodes were connected through Ag/AgCl wires and a guarded coaxial cable to a high impedance ($>10^{15}$ Ω) FET-input electrometer (Analog Devices 515L) with capacitance neutralization. The amount of capacitance neutralized was adjusted to reduce to less than 0.2 pF the input line capacitance. A gated 5 V source in series with a 10^{10} Ω resistor was used to apply 0.5 nA depolarizing current pulses (I_M) through the open-tip microelectrodes in order to monitor their resistance (R_{el}). An electronic current-clamp device, was used to control the I_T and I_M pulses and to calculate transepithelial resistance R_T, and the fractional apical voltage ratio fV_a. The latter is the ratio between the deflections in apical membrane potential ΔV_a, and transepithelial potential ΔV_T, produced by the I_T pulse[2]. When using conventional microelectrodes, I_T and I_M pulses were applied alternatively. Each pulse had a duration of 1.1 sec and the interval between individual pulses was 2.2 sec. R_T was electronically calculated from the deflections in V_T produced by the I_T pulses. A divider (Analog Devices 533D) was used to calculate fV_a from the deflection in V_a (or V_{Cl}) and V_T produced by the I_T pulses. Both R_T and fV_a were displayed, with the use of sample-and-hold circuits, in two panel meters. Two additional panel meters were used to display V_T and either V_a or V_{Cl}. In addition, R_T, fV_a, V_T and V_a (or V_{Cl}), were recorded in four channels of a strip chart recorder (Gould-Brush, Mark 240).

[2] We prefer to denote this ratio by the symbol fV_a since only under special circumstances is fV_a equal to the fractional resistance of the apical membrane, i.e. $fR_a \equiv R_a/(R_a + R_b)$, where R_a and R_b are the equivalent input resistances of the apical and basolateral membranes (Boulpaep & Sackin, 1980).

When using Cl^--selective microelectrodes, only the I_T pulses were applied. Also, because of the long time constants of some of these microelectrodes, the duration of these pulses and the interval between them were increased to 4.4 and 14 s, respectively, to allow a full response of the microelectrode potential[3]. Throughout these experiments the intensity of the I_T pulse was 15 μA (current density 39.5 μA/cm^2), in the serosal-to-mucosal direction. The electronic device provided compensation of R_T and fV_a for the resistance of the external solutions. All potentials were measured with reference to the mucosal solution.

Measured changes in potential following alterations in the composition of either the mucosal or the serosal solution were corrected for the asymmetric liquid junction potentials arising at the tip of the V_T measuring bridges. These junction potentials were measured as follows: The agar bridge connecting the calomel cell to the compartment where the solution was changed contained the initial Ringer's solution (Ringer A). The contralateral bridge was 3 M KCl-agar throughout the experiment. The junction potential that arose when Ringer B was substituted for Ringer A was measured in the circuit:

calomel | 3 M KCl | agar-Ringer A | Ringer B |
V_1 V V_2
agar-3 M KCl | 3 M KCl | calomel.

In this situation one measures the true junction potential (V) plus an error ($V_1 + V_2$) due to the asymmetric junction potentials between 3 M KCl and each of the two Ringer's solutions. This error is expected to be about 2 to 3 mV (Barry & Diamond, 1970; Frizzell & Schultz, 1972). When the mucosal (reference) solution was changed, both V_T and V_a were corrected for junction potentials. For changes in the serosal solution, V_T only need be corrected. For changes in pH, junction potentials were less than 0.5 mV. This small change was probably due to the different buffers employed. Substitution of 90 mM gluconate or methylsulfate for Cl^- generated junction potentials of −9.5 and −5.5 mV, respectively. Junction potential measurements were repeated several times before and after an experiment and always gave values that were constant within 0.3 mV.

Experimental Procedure

Microelectrodes were advanced perpendicularly to the tissue using a piezoelectric positioning device (Inchworm, Burleigh Instruments) attached to a micromanipulator (MM-33, Narishige). Initially, the micromanipulator was used to position the microelectrode close to the tissue. This was done under microscopic observation through a window in the chamber. The cells were then impaled through the mucosal membrane by advancing the piezoelectric device in steps of 4 to 6 μm. On some occasions an additional 2 μm advance or withdrawal of the microelectrode was necessary following impalement in order to obtain a stable intracellular potential with no change in R_{el}. Specific details of the acceptability of impalements are described below. The experimental protocol was as follows: Following a minimum of five acceptable impalements with a conventional and a Cl^--selective microelectrode, either the mucosal or the serosal solution was changed to one with a different pH, while keeping one of these microelectrodes inside a cell. When the microelectrode potential reached a stable value, the original solution was reintroduced. Several such changes were made with a conventional and a Cl^--selective microelectrode alternatively inside a cell. Following this, the second solution was allowed to perfuse for at least 40 min. Further sets of impalements were then made with conventional and Cl^--selective microelectrodes. In the Cl^--substitution experiments, all changes in V_a and other parameters were recorded during single-cell impalements.

[3] The longer I_T pulses employed with Cl^--selective microelectrodes occasionally produced tissue polarization. When this occurred, the fractional apical voltage ratio obtained with Cl^--selective microelectrodes was somewhat smaller than the values obtained with conventional microelectrodes. Therefore, only the latter values are reported here. In a few cases where the long pulse was employed for both types of microelectrodes, the values obtained were identical.

Results

The criteria for the acceptance of microelectrode impalements employed in a previous study (Garcia-Diaz & Armstrong, 1980), were complemented in the present experiments by the use of two additional criteria based on the measurements of R_{el} and fV_a. Continuous recording of R_{el} is important for the accurate determination of V_a since plugging of the microelectrode tip by membrane or intracellular components (seen as an increase in R_{el} following impalement) is often associated with an artifactual hyperpolarization (Armstrong & Garcia-Diaz, 1981). When this happened it was possible in most cases to reduce R_{el} to its value before impalement by moving the microelectrode in 2-μm steps, usually in the direction of withdrawal. When this was done, V_a, which was initially unstable and showed occasional periodic oscillations, settled down to a stable value sometimes a few mV lower than the original value following impalement.

Measurement of fV_a, with conventional and Cl^--selective microelectrodes, is useful to determine if the membrane has been irreversibly damaged by penetration with the microelectrode. Since shunting of the apical membrane upon impalement will artifactually decrease the deflection in V_a (or V_{Cl}) produced by transepithelial current pulses, recordings that showed either a continuous decline of fV_a after impalement or a value of fV_a significantly lower than those obtained during other impalements with the same type of microelectrode (i.e. conventional or Cl^--selective) in the same preparation were rejected. When fV_a declined after penetration of the cell, V_a (or V_{Cl}) depolarized in a parallel fashion. In some instances, following an initial decline, V_a slowly repolarized and fV_a increased simultaneously. This could occur spontaneously or after a further advance of the microelectrode. When both parameters eventually reached stable values that were equal to or higher than those observed immediately following impalement, the recording was considered acceptable.

With identical solutions bathing both sides of the bladder, V_T was ≤ 2 mV. During the initial 10

to 30 min after mounting the tissue, fV_a and V_a decreased exponentially and R_T increased. In most cases, a steady state was reached after this initial period, and, thereafter, V_a, fV_a and R_T remained almost constant for several hours. Only experiments in which these parameters had stabilized in this fashion were used to study longterm effects of pH changes. The initial decreases in V_a and fV_a paralleled each other, as shown by the linear relation of Fig. 1*A*. A similar relationship was observed between the steady-state values of V_a and fV_a in different gallbladders (Fig. 1*B*).

Changes in Solution pH

Figure 2 shows the results of an experiment in which mucosal pH was changed between 8.2 and 7.2 while an open-tip (*a*) or a Cl^--selective microelectrode (*b*) was kept inside a cell. Perfusion with the more alkaline solution (periods indicated by dashed bars) hyperpolarized V_a and V_{Cl} by 6 and 7 mV, respectively. At the same time fV_a decreased by 0.1 and R_T increased slightly (10 Ω cm^2). The small change in V_T seen in Fig. 2 is largely due to the uncorrected junction potential (0.3 mV). The changes illustrated in Fig. 2 were reversible and consistent throughout a given experiment. The results of all experiments of this kind are summarized in the top four rows of Table 1. The changes in V_a and V_{Cl} did not differ significantly from each other.

In another set of experiments the pH of the serosal solution was changed. Figure 3 shows that when serosal pH was increased from 7.2 to 8.2 (dashed bar) V_a hyperpolarized by 10 mV, fV_a increased from 0.5 to 0.6 and R_T decreased by about 20 Ω cm^2. In this experiment V_T increased by 0.5 mV.

Figure 4 shows the response of V_{Cl} to changes in the pH of both solutions. Initially both sides of the epithelium were bathed with solutions at pH 8.2. When the mucosal pH was decreased to 7.2 (solid bar) V_{Cl} depolarized by 7 mV. Upon restoring the initial mucosal pH, V_{Cl} increased toward the original value. Decreasing the serosal pH to 7.2 (end of dashed bar) slowly depolarized V_{Cl} by 9 mV. Reduction of mucosal pH to 7.2 (solid bar) further depolarized V_{Cl} by 6 mV. This and other similar experiments showed that the effects of mucosal and serosal pH changes on V_a and V_{Cl} were additive (*see also* Fig. 5). The results of serosal pH changes are shown in the last four rows of Table 1.

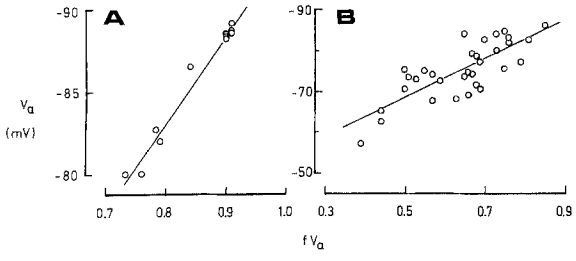

Fig. 1. *A*. Relationship between V_a and fV_a in a single gallbladder during the initial 40 min after mounting the tissue. Each point represents an individual acceptable impalement. The first impalement is the uppermost point on the right-hand side of the graph. The last one (representing the steady state) is the extreme left-hand in the graph. Solid line is the least-squares regression line ($y=53x+41$, $r=0.99$, $P<0.001$). *B*. Relationship between V_a and fV_a for 36 gallbladders perfused with symmetrical solutions at pH 8.2. Each point represents the mean V_a and fV_a of each gallbladder after a steady state was reached. Solid line is the least-squares regression line ($y=46.5x+45$, $r=0.78$, $P<0.001$)

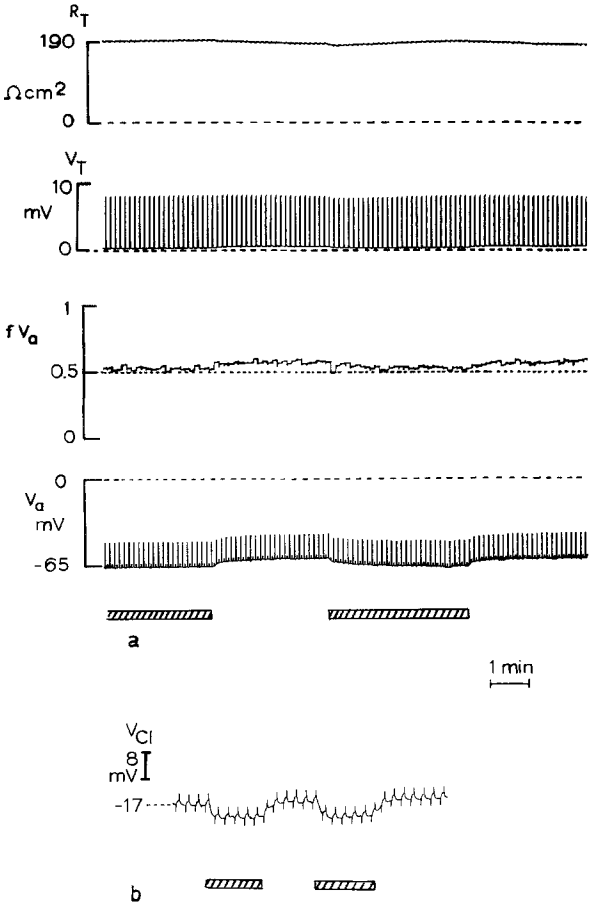

Fig. 2. Effects produced by mucosal pH changes during single-cell impalements. During the period marked by the dashed bars the mucosal pH was 8.2. Serosal pH was always 7.2. The small deflections in the V_a trace and the deflections in the V_T and V_{Cl} traces are due to transepithelial current pulses (39.5 μA/cm^2). The large deflections in the V_a recording are produced by 0.5 nA microelectrode current pulses

Table 1 shows that increasing the pH of either the mucosal or serosal solution hyperpolarizes V_a and V_{Cl}, with no significant change in V_T. The hyperpolarization is larger when the pH of the serosal solution is increased. With both experimental maneuvers, the change in V_a is not significantly different from the change in V_{Cl}. This indicates that intracellular Cl^- activity, a^i_{Cl}, remains constant. The direction of the changes in R_T and fV_a when the mucosal pH is raised is opposite to that observed when the pH of the serosal solution is increased.

Reuss, Cheung and Grady (1981) found that the hyperpolarization in V_a produced by an increase in mucosal pH is mainly due to an increased K^+ conductance of the apical membrane. If this is the only effect of a change in pH and if the cell membrane allows electrodiffusive movement of Cl^-, one would predict a decrease in a^i_{Cl} following hyperpolarization of V_a. The finding that a^i_{Cl} remains constant during relatively short periods

Table 1. Effect of external pH on electrophysiological parameters of *Necturus* gallbladder

	R_T (Ω cm^2)	fV_a	V_T (mV)	V_a (mV)	V_{Cl} (mV)
Mucosal pH:					
7.2	190±60	0.54±0.10	0.3±0.6	−61±5	−20±8
8.2	210±60	0.49±0.12	0.3±0.6	−67±7	−25±10
Δ	20±10	−0.05±0.03	0 ±0.3	− 6±3	− 5±3
P	<0.001	<0.001	NS	<0.001	<0.001
Serosal pH:					
7.2	150±20	0.55±0.10	0.4±0.4	−62±7	−24±8
8.2	130±20	0.61±0.10	0.3±0.3	−72±6	−33±7
Δ	−20±20	0.06±0.03	0.1±0.3	−10±3	− 9±4
P	<0.01	<0.001	NS	<0.001	<0.01

Mean ±SD. P values from paired Student's t-test. Contralateral pH maintained at 7.2. Mucosal pH: $n=17$, in seven animals for all data except for V_{Cl} ($n=12$, in five animals). Serosal pH: $n=13$, in four animals for all data except for V_{Cl} ($n=5$, in three animals).

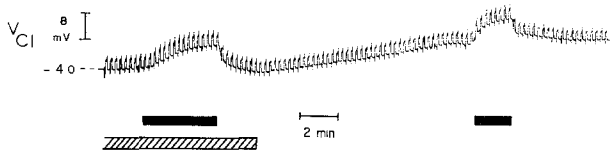

Fig. 4. Effects on V_{Cl} of changes in mucosal and serosal pH. During the period marked by the dashed bar the serosal pH was 8.2. At the end of the dashed bar it was changed to 7.2. Solid bars: mucosal pH decreased from 8.2 to 7.2

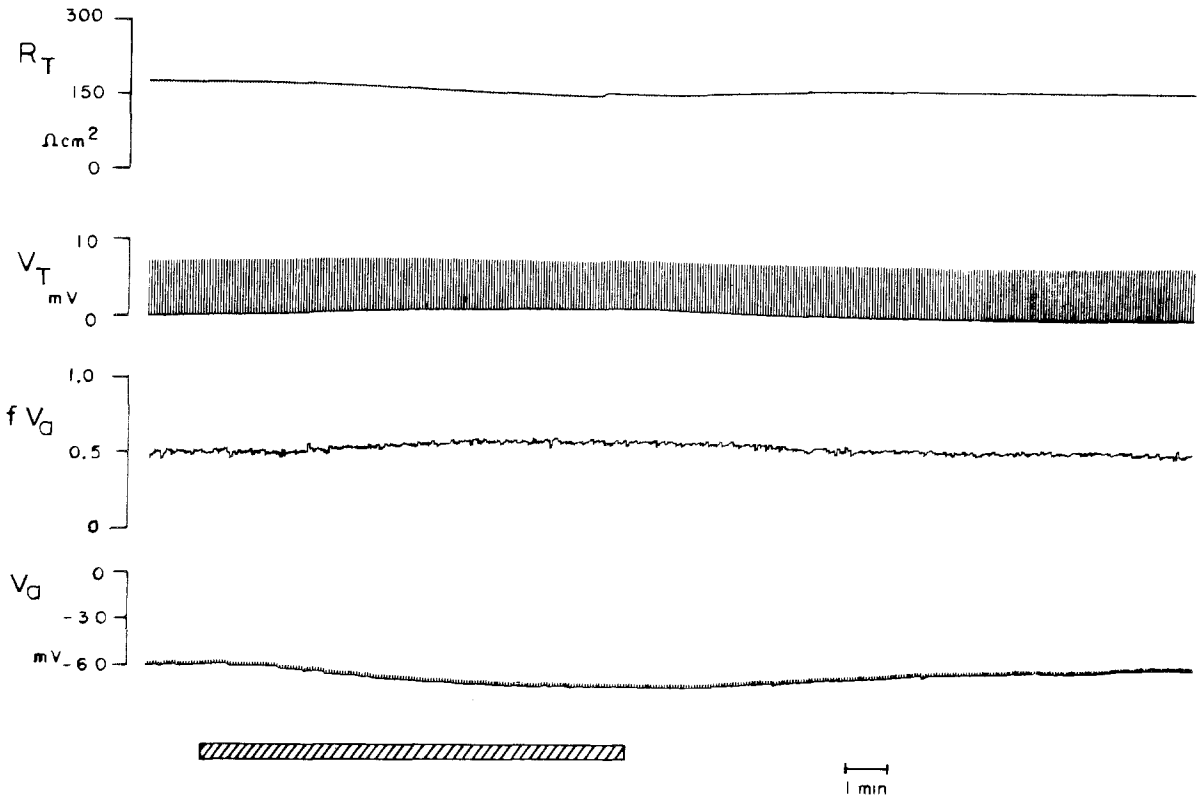

Fig. 3. Effects produced by serosal pH changes during single-cell impalement. During the period marked by the dashed bar the serosal pH was increased from 7.2 to 8.2. Mucosal pH was maintained at 7.2 throughout the experiment. The deflections produced in the V_a trace by transepithelial and microelectrode current pulses are not distinguishable from each other in this Figure

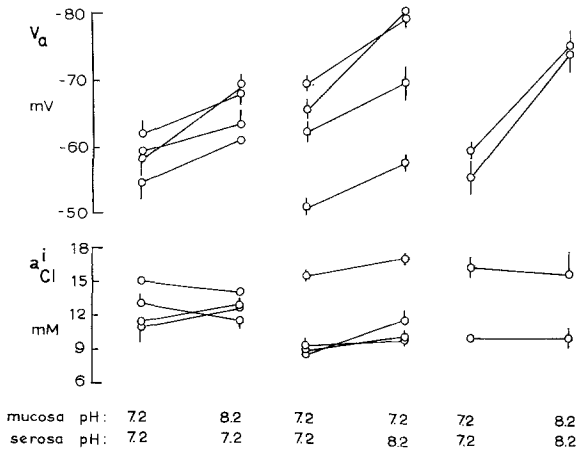

Fig. 5. Effects of unilateral or bilateral pH changes on the steady-state apical membrane potential (V_a) and intracellular Cl^- activity (a^i_{Cl}). Individual gallbladders are connected by lines. Vertical lines represent ±SD

($\leq$10 min) of hyperpolarization could be a consequence of a low Cl^- permeability at the cell membrane. To see if a^i_{Cl} changed after a sufficiently long-term hyperpolarization of the membrane potential, we measured steady-state values of V_a and a^i_{Cl} in the same preparation at pH 7.2 and 8.2. As described under Materials and Methods, when the pH was changed in these experiments, at least 40 min were allowed for the establishment of a new steady state. The results obtained are shown in Fig. 5. As seen in the short-term experiments already described, increasing the pH of the mucosal or serosal solutions hyperpolarized V_a. The average hyperpolarizations observed were 6 and 10 mV, respectively. In two experiments where the pH of both solutions was changed, V_a hyperpolarized by approximately 17 mV, again indicating that the effects of increasing mucosal and serosal pH were additive. However, a^i_{Cl} did not change significantly under any of the above conditions.

The insensitivity of a^i_{Cl} to changes in V_a is further illustrated by the results shown in Table 2. This Table lists the mean V_a and a^i_{Cl} values from the 10 gallbladders included in Fig. 5 when both bathing solutions are at pH 7.2, together with the values measured in 21 preparations bathed by symmetrical solutions at pH 8.2. In agreement with the results already presented, V_a is 16 mV higher at pH 8.2 than at pH 7.2 but a^i_{Cl} does not differ significantly between the two experimental groups. Also shown in Table 2 is the ratio (R) between a^i_{Cl} and the equilibrium Cl^- activity, that is the value of a^i_{Cl} predicted from the Nernst equation on the assumption that Cl^- is at electrochemical equilibrium across the cell membrane. Because of

Table 2. Dependence of apical membrane potential (V_a) and intracellular Cl^- activity (a^i_{Cl}) on external pH

	V_a (mV)	a^i_{Cl} (mM)	R	n
pH 7.2	−60±5	12±2	1.5	10
pH 8.2	−76±6	13±3	3.0	21
P	<0.001	NS		

Both mucosal and serosal solutions at the same pH. R: Ratio of a^i_{Cl} to the equilibrium Cl^- activity. n: number of gallbladders. Means±SD

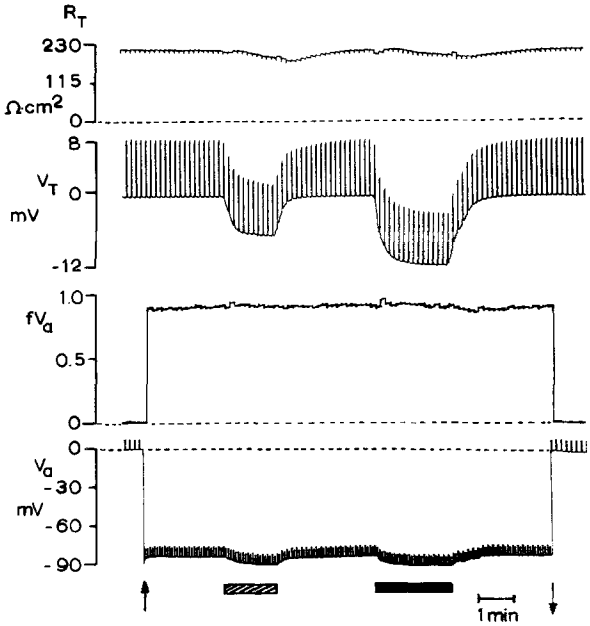

Fig. 6. Effects of luminal substitution of 90 mM Na-methylsulfate (dashed bar) or Na-gluconate (solid bar) for NaCl during single-cell impalement. Changes in V_T and V_a are not corrected for junction potential. The pH was 8.2 throughout this experiment. The arrows indicate impalement (↑) or withdrawal (↓) of the microelectrode. The deflections in V_T and the smaller deflections in V_a represent transepithelial current pulses (39.5 μA/cm²). The larger deflections in V_a are due to the microelectrode current pulses (0.5 nA). Note that only these are seen when the microelectrode is outside the cell

the lower equilibrium Cl^- activity corresponding to the more negative V_a, this ratio is twice as large at pH 8.2 than at pH 7.2. The implications of this increase in R will be discussed later.

Mucosal Cl^- Substitution

Although, as discussed later, there may be alternative explanations, the above results are probably the consequence of a very low electrodiffusive permeability of the apical and basolateral cell membranes to Cl^- ions. If this is so, changes in the external Cl^- activity should not affect the intrinsic

Table 3. Effects of luminal Cl^- substitutions

	pH = 8.2				pH = 7.2
	R_T (Ω cm^2)	fV_a	V_T (mV)	V_a (mV)	V_a (mV)
Control (NaCl)	180±30	0.69±0.10	1.0±1.0	−75±8	−68±8
Na-gluconate	190±30	0.66±0.14	−0.6±1.3	−75±7	−84±7
Δ	10±20	−0.03±0.07	−1.6±1.0	0±1	16±6
P	NS	NS	<0.01	NS	<0.001
Na-$MeSO_4$	180±30	0.67±0.12	0.3±0.8	−76±6	−79±7
Δ	0±10	−0.02±0.03	−0.7±0.3	− 1±3	−11±5
P	NS	NS	<0.01	NS	<0.001

Means ± SD for seven gallbladders. There are at least four substitutions by each anion in each gallbladder. Paired Student's t-test.

electromotive forces of these membranes. In his studies of the ionic permeability of *Necturus* gallbladder, Reuss (1979) found an extremely low permeability for Cl^- at the basolateral cell membrane. On the other hand, the situation appears to be less conclusive with respect to the apical membrane. In one study (Reuss & Finn, 1975*b*) it was reported that V_a depolarized following substitution of luminal Cl^- by other anions. In another (van Os & Slegers, 1975) it was found that V_a remained constant or hyperpolarized slightly under these conditions. Therefore we investigated the effects of mucosal Cl^- substitution on the electrical parameters of *Necturus* gallbladder.

Figure 6 shows an experiment where 90 mM Na-methylsulfate (dashed bar) or 90 mM Na-gluconate (solid bar) were substituted for an equivalent amount of NaCl in the mucosal solution. The pH of both bathing solutions was kept at 8.2 throughout this experiment. This Figure shows data obtained directly from the strip-chart recorder, without correction for the junction potential at the mucosal bridge that makes V_T and V_a appear more negative than their actual values during Cl^- substitution. After correction, V_T was 0.6 mV and 1.1 mV more negative during substitution of methylsulfate or gluconate, respectively, for Cl^-. V_a hyperpolarized by 0.5 mV with methylsulfate and depolarized by 1.7 mV with gluconate. R_T decreased slightly during partial removal of Cl^- but fV_a did not change. The small changes in V_a and R_T seen in this particular experiment were not observed in all the gallbladders studied. Table 3 shows the results from seven preparations at pH 8.2. Only the effect on V_T is significant. Since changes in V_T primarily reflect the behavior of the paracellular pathway diffusion potential (van Os & Slegers, 1975; Reuss & Finn, 1975*a, b*), the more negative V_T after the substitution of Cl^- seems to indicate that gluconate and methylsulfate are slightly more permeant than Cl^- across the paracellular pathway. However, as will be discussed later, the uncertainty in the measurement of the junction potential may be responsible for this apparent permselectivity sequence. A factor that may contribute to the insensitivity of V_a to luminal Cl^- is the high K^+ permeability of the apical membrane that dominates the behavior of V_a. In an attempt to assess the contribution of Cl^- ions to the apical membrane emf we repeated the Cl^- substitution experiments at pH 7.2, that is, when apical K^+ permeability is lower than at pH 8.2 (Reuss et al., 1981). At pH 7.2 the most significant effect was a hyperpolarization of V_a. This is shown in the last column of Table 3. Assuming that the apical membrane is impermeable to methylsulfate and gluconate, this observation can only be explained by an effect of Cl^- on the cationic permeability of the apical membrane, e.g. an increase in P_K or decrease in P_{Na} when mucosal Cl^- is reduced.

Because of the extracellular shunt pathway, the behavior of V_a in *Necturus* gallbladder does not directly reflect that of the emf (E_a) across the apical cell membrane. However, as outlined below, it is possible to estimate the effect of luminal Cl^- substitution on E_a from the analysis of the lumped electrical equivalent circuit (Reuss & Finn, 1975*a*; van Os & Slegers, 1975). Unfortunately, the dependance of the apical membrane cation permeability on mucosal Cl^- concentration suggested by the present results limits the accuracy of this estimate.

Discussion

Although Fromm and Schultz (1981) measured a significant rate of KCl leakage from microelectrodes similar to those employed herein, our measurements of V_a do not seem to be affected by KCl

leakage. First, in those impalements where damage to the cell membrane or plugging of the tip were not apparent upon impalement (as indicated by the microelectrode resistance and fractional voltage ratio, *see* Results), V_a remained constant for periods of up to 30 min. Second, in a given preparation, the same values of V_a were recorded when the microelectrodes were backfilled with 1 or 3 M KCl. As suggested by Fromm and Schultz (1981) this may be due to the syncytial character of the epithelium. Low resistance cell-to-cell coupling allows rapid diffusion of KCl and the maintenance of a constant intracellular potential.

In the lumped equivalent circuit used to represent the electrical properties of epithelia (Reuss & Finn, 1975*a*; van Os & Slegers, 1975), the ratio fV_a, between the deflections in the apical membrane potential and the transepithelial potential in response to a current pulse is equal to the fractional resistance of the apical membrane fR_a, where $fR_a = R_a/(R_a + R_b)$. Boulpaep and Sackin (1980) have shown that the lumped equivalent circuit is only an approximation. In particular, the distributed model employed by these authors predicts that when the resistance of the lateral intercellular spaces contributes significantly to the total resistance of the extracellular pathway, the ratio fV_a will be smaller than fR_a. The larger the contribution of the lateral interspaces to the shunt resistance, the greater will be the difference between fV_a and fR_a. This is particularly important in leaky epithelia with low junctional resistances. However, the use of fV_a to assess membrane damage is valid, independently of the model employed to represent the epithelium.

The V_a measured at pH 7.2 in the present experiments (mean value, −60 mV) is higher than the value (−52 mV) previously reported from this laboratory (Garcia-Diaz & Armstrong, 1980). This is due to the lower external K^+ concentration (2.5 mM against 5.4 mM) used in the present study and the high P_K of the *Necturus* gallbladder epithelial cells (Reuss & Finn, 1975*a*). The higher V_a measured at pH 8.2 (−76 mV) agrees with the value (−78 mV) obtained by Reuss et al. (1981) using a solution with a pH and ionic composition similar to those employed in this study.

The relationship between fV_a and V_a presented in Fig. 1*B* was also observed by Graf and Giebisch (1979) in *Necturus* gallbladder with impalements that were validated by satisfactory criteria. Our conclusion that the relationships shown in Fig. 1*A* and *B* are not a consequence of membrane damage is based on the following observations: 1) All impalements that were accepted conformed to the stability criteria previously outlined. 2) For each preparation shown in Fig. 1*B* the scatter among the steady-state values of fV_a and V_a obtained in individual impalements was minimal, even when microelectrodes of different resistance were employed. It seems highly improbable that different penetrations would suffer the same degree of shunting due to membrane damage. 3) A similar argument can be applied to the data obtained in a given preparation during relaxation toward a steady state. If the variations in fV_a and V_a observed in experiments such as the one shown in Fig. 1A were caused by damage to the apical membrane, one would expect high and low values of V_a and fV_a to be randomly distributed in time. The uniform temporal dependence of these parameters throughout our experiments argues against the possibility that the relationship illustrated in Fig. 1*A* is due to membrane damage.

The actual mechanism responsible for the variations in fV_a and V_a observed in Figs. 1*A* and *B* is not clear. Although changes in the lateral space width will alter fV_a in the absence of any real change in the resistance of the cell membranes (Boulpaep & Sackin, 1980), this would not significantly affect V_a. Graf and Lewis (*see* Appendix in Graf & Giebisch, 1980) assumed that the variations in fV_a and V_a reflected primarily changes in the apical membrane resistance. However, the observation that, in experiments like the one shown in Fig. 1*A*, R_T increased at the same time that fV_a and V_a decreased, may indicate that the latter reflect an increase in the basolateral membrane resistance. In the absence of more detailed knowledge concerning the behavior of the apical and basolateral membrane emf's and resistances, it is not possible to ascertain the relative contributions of each of these membranes to the changes in fV_a and V_a.

The effect of mucosal pH on the electrical parameters of *Necturus* gallbladder has been described by Reuss et al. (1981). Mucosal acidification produced a depolarization in V_a that was explained by a decrease in the K^+ permeability, P_K, of the apical membrane. These authors suggested that this effect is mediated by titration of membrane fixed negative charges and a specific effect of luminal proton activity on the apical K^+ channels (Gögelein & Van Driessche, 1981). The magnitude and direction of the change in V_a observed in the present experiments agree with those reported by Reuss et al. (1981) between pH 7 and pH 8.

As found by Reuss et al. (1981), changes in pH did not significantly affect V_T in the present study. Increasing the mucosal pH resulted in a small but

significant decrease in fV_a (*see* Fig. 2 and Table 1). This agrees with the idea of a higher cation conductance of the luminal membrane at lower luminal proton activity. Reuss et al. (1981) found, however, that the direction of the change in fV_a varied between different preparations. These authors also found that R_T decreased slightly between pH 7 and 8. In the present experiments R_T increased when the mucosal solution pH was raised from 7.2 to 8.2. The reason for this discrepancy is not yet clear.

The results obtained with alkalinization of the serosal solution, namely a hyperpolarization of V_a and an increase in fV_a are more difficult to explain. An analysis of these results is greatly facilitated by considering the lumped equivalent circuit for gallbladder epithelium (*see* Reuss & Finn, 1975*a*; van Os & Slegers, 1975). In this circuit, the transepithelial potential difference V_T is given by

$$V_T = E_b - E_a + I_c(R_a + R_b) \quad (1)$$

where E_a and R_a are the electromotive force (zero current potential) and lumped resistance of the apical membrane, E_b and R_b are the corresponding parameters for the basolateral membrane, and I_C is the (serosal-to-mucosal) current through the cellular pathway. E_a and E_b are both oriented with the negative pole inside the cell. The apical membrane potential is

$$V_a = -E_a + I_c R_a. \quad (2)$$

Substituting I_c from Eq. (1) in Eq. (2) and rearranging,

$$-V_a = E_a + fR_a(E_b - E_a - V_T) \quad (3)$$

where $fR_a = R_a/(R_a + R_b)$. In principle, changes in the basolateral solution could affect V_a either through E_b or R_b. Since the basolateral membrane behaves virtually as an ideal K^+-selective membrane (van Os & Slegers, 1975; Reuss, 1979), E_b is close to the K^+ equilibrium potential. Therefore if, as happens at the apical membrane, P_K increases following alkalinization of the serosal solution, E_b will hardly be affected. On the other hand, an increase in P_K will reduce R_b with a consequent increase in fR_a and hyperpolarization of V_a (*see* Eq. 3). The question is whether the fall in R_b is large enough to account for the 10 mV hyperpolarization of V_a under these conditions. If one assumes that the measured change in fV_a (0.06) accurately reflects the increase in fR_a, it can be calculated from Eq. (3) that a hyperpolarization of 10 mV in V_a requires a value of 165 mV for $(E_b - E_a)$. This is obviously too high, since estimates of E_b alone range from 75 to 90 mV (Reuss & Weinman, 1979). A possible explanation for this discrepancy is that the measured change in fV_a underestimates (because of the distribution of resistance along the lateral space) the increase in fR_a. If one assumes $(E_b - E_a)$ to be about 50 mV (Reuss & Weinmann, 1979), it can be shown from Eq. (3) that the increase in fR_a must be 0.2, that is a value more than 3 times the measured change in fV_a. On the basis of the present experiments, it is not possible to ascertain the exact mechanism that gives rise to the hyperpolarization in V_a.

Under all conditions studied, the changes in V_a produced by alterations in the medium pH were paralleled by equal changes in V_{Cl}. Thus, a^i_{Cl} remained constant despite significant changes in V_a. In principle, all other parameters being equal, one would expect a decrease in a^i_{Cl} following hyperpolarization of V_a if the cell membranes of *Necturus* gallbladder allow electrodiffusive movement of Cl^-. Insensitivity of a^i_{Cl} to changes in membrane potential has been also shown in the proximal tubule of *Necturus* (Spring & Kimura, 1978; Shindo & Spring, 1981). This result has been taken as evidence for a negligible Cl^- conductance at the cell membranes of this tissue. Similar results were reported recently by Guggino et al. (1982). This also seems to be the case in *Necturus* gallbladder. Reuss (1979) and van Os and Slegers (1975) found Cl^- conductance at the basolateral membrane to be extremely low or absent. As discussed below, the experiments on luminal Cl^- substitution reported herein support the notion of a negligible apical Cl^- conductance.

An alternative explanation for the constancy of a^i_{Cl} is that P_{Cl} is reduced at the higher pH in a way that compensates for the increased driving force for Cl^- exit. Smith, Orellana and Field (1979) obtained indirect evidence for a decrease of luminal membrane P_{Cl} in the intestine of winter flounder when medium pH was increased. This could reflect a voltage-dependent P_{Cl}. Reuss and Grady (1979) found that depolarization of *Necturus* gallbladder cells following substitution of K^+ for Na^+ in the luminal medium increased a^i_{Cl}, even when mucosal Cl^- was replaced by SO_4^{2-}. To account for the rate of Cl^- entry across the basolateral membrane of the depolarized cells they had to assume that P_{Cl} at this membrane increases after depolarization. However, the mechanism responsible for the increase in Cl^- entry could be other than electrodiffusive (e.g. coupled KCl movement). It seems unlikely that a voltage-dependent P_{Cl} could account for the present results. Such an explanation would require reductions in P_{Cl} that would exactly compensate for hyperpolarizations in V_a of -6, -10, and -17 mV, respectively, fol-

lowing pH increases in the mucosal, the serosal, or both media. Thus, although the possibility of a voltage-dependent P_{Cl} cannot be completely rejected on the basis of the present experiments, the most plausible explanation for our results is the lack of a significant electrodiffusive Cl^- permeability at both cell membranes.

The Cl^- substitution experiments reported herein support the above interpretation, since they indicate that Cl^- ions do not contribute significantly to the emf of the apical membrane, E_a. To assess the magnitude of the effect of Cl^- removal on E_a, Eq. (3) can be rewritten as:

$$E_a = \alpha(V_T - E_b) - (\alpha + 1)\, V_a \tag{4}$$

where $\alpha = R_a/R_b = fR_a/(1 - fR_a)$. Two assumptions can now be made. First, it is reasonable to ignore possible effects on the basolateral membrane parameters R_b and E_b during rapid changes in the ionic composition of the mucosal solution (Reuss & Finn, 1975*a*). The second assumption is that the reduction in mucosal Cl^- concentration does not affect R_a. This is not unreasonable since, as shown in Fig. 6 and Table 3, fV_a did not change following removal of luminal Cl^- at pH 8.2. Also, Reuss and Finn (1975*b*) did not find a significant change in R_a under conditions similar to those used in the present experiments. Thus, the change in E_a upon reduction in external Cl^- is given by:

$$\Delta E_a = \alpha \Delta V_T - (\alpha + 1)\, \Delta V_a. \tag{5}$$

With the approximation $fV_a \simeq fR_a$, ΔE_a can be calculated from the data in Table 3 at pH = 8.2, where $\alpha = 2.2$ and $\Delta V_a = 0$. The results obtained are depolarizations of 3.5 mV with gluconate and 1.5 mV with methylsulfate. These, in turn, give values of 0.074 and 0.032 for the Cl^- transport number t_{Cl} across the apical cell membrane. However, these estimates are subject to serious error because of the uncertainty in measuring the junction potential at the tip of the agar bridge that makes contact with the luminal solution. This uncertainty can amount to 2 to 3 mV (Barry & Diamond, 1970) and could be the cause of the observed change in V_T. Taken at its face value, this change in V_T gives the apparent permselectivity sequence $P_{Cl} < P_{MeSO_4} < P_{gluconate}$ for the paracellular pathway, i.e. permselectivity appears to be inversely related to the size of the anion. This anomalous result strongly suggests that the effect of the uncertainty in the junction potential is to increase the value of ΔE_a calculated from Eq. (5). Hence, it seems justifiable to conclude that the true value of t_{Cl} is very small and that no appreciable contribution of Cl^- ions to E_a was demonstrated in the present experiments. The large hyperpolarization in V_a following replacement of luminal Cl^- at pH 7.2 can be explained only as an effect of Cl^- removal on the cation permeability of the apical membrane, that is, a reduction in P_{Na} or an increase in P_K, such as that reported by Krasny, Halm and Frizzell (1982) for flounder intestine and Guggino et al. (1982) for *Necturus* proximal tubule. The results of the substitution experiments at different pH values suggest that the latter possibility is more likely. This dependance of cation permeability on luminal Cl^- concentration makes the above calculation of t_{Cl} still more uncertain. It is possible that reduction in luminal Cl^- concentration increases P_K even at pH 8.2. The associated hyperpolarization of E_a could mask part of the depolarization expected from a finite P_{Cl}.

In agreement with these observations, Van Os and Slegers (1975) found a small hyperpolarization of V_a after luminal substitution of SO_4^{2-} for Cl^- at pH 7.4, for which they did not have an explanation. On the other hand, Reuss and Finn (1975*b*) found a depolarization in V_a and a large serosal negative change in V_T following mucosal Cl^- substitution. The reason for the discrepancy between the results of Reuss and Finn (1975*b*) and the present findings is not clear, although it could be due at least in part, to the method employed by these authors to measure the junction potential. To do this, they used a Ringer's-agar bridge (instead of 3 M KCl-agar) to connect the compartments containing the two different Ringer's solutions. It is clear from a consideration of the resulting circuit that this introduces a new junction potential that opposes the one being measured and may in fact completely mask it. Hence, the true junction potential will be, at the very least, seriously underestimated. However, this cannot explain the depolarization in V_a reported by these investigators since underestimating the junction potential would result in a larger apparent apical membrane hyperpolarization.

In summary, the experiments presented herein indicate that the electrodiffusive permeability of the apical membrane to Cl^- is negligibly small. Therefore, Cl^- transport at this membrane is, in large measure, an electroneutral process either in the form of a coupled NaCl mechanism or of a double exchange (Na^+/H^+ and Cl^-/HCO_3^-). Since the Cl^- conductance of the basolateral membrane is also much too low to account for the observed rates of Cl^- transport, Cl^- movement at this membrane has been postulated to be largely electroneutral, e.g. coupled KCl exit or Cl^-/HCO_3^- exchange (Reuss, 1979).

Finally, it is important to point out that, as a consequence of the very low electrodiffusive permeability of the cell membranes to Cl^-, the use of the ratio (R) of a^i_{Cl} to the equilibrium Cl^- activity to evaluate transcellular Cl^- transport under different experimental conditions is of little value. This is clearly shown by the results of Table 2. The steady-state value of R at pH 8.2 is twice that observed at pH 7.2. However, this is merely a consequence of the higher V_a at pH 8.2. It does not imply any change on the mechanisms responsible for Cl^- transport. Intracellular Cl^- activity would appear to be a more appropriate index of transcellular Cl^- transport.[4]

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