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Effects of External Sodium and Cell Membrane Potential on Intracellular Chloride Activity in Gallbladder Epithelium

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Summary. Conventional and Cl-selective liquid ion-exchanger intracellular microelectrodes were employed to study the effects of extracellular ionic substitutions on intracellular Cl activity (aCl_i) in *Necturus* gallbladder epithelium. As shown previously (Reuss, L., Weinman, S.A., 1979; *J. Membrane Biol.* **49**:345), when the tissue was exposed to NaCl-Ringer on both sides aCl_i was about 30 mM, i.e., much higher than the activity predicted from equilibrium distribution (aCl_{eq}) across either membrane (5–9 mM). Removal of Cl from the apical side caused a reversible decrease of aCl_i towards the equilibrium value across the basolateral membrane. A new steady-state aCl_i was reached in about 10 min. Removal of Na from the mucosal medium or from both media also caused reversible decreases of aCl_i when Li, choline, tetramethylammonium or N-methyl-D-glucamine (NMDG) were employed to replace Na. During bilateral Na substitutions with choline the cells depolarized significantly. However, no change of cell potential was observed when NMDG was employed as Na substitute. Na replacements with choline or NMDG on the serosal side only did not change aCl_i. When K substituted for mucosal Na, the cells depolarized and aCl_i rose significantly. Combinations of K for Na and Cl for SO_4 substitutions showed that net Cl entry during cell depolarization can take place across either membrane. The increase of aCl_i in depolarized cells exposed to K_2SO_4-Ringer on the mucosal side indicates that the basolateral membrane Cl permeability (P_{Cl}) increased. These results support the hypothesis that NaCl entry at the apical membrane occurs by an electroneutral mechanism, driven by the Na electrochemical gradient. In addition, we suggest that Cl entry during cell depolarization is downhill and involves an increase of basolateral membrane P_{Cl}.

Cl transport across the luminal membrane of *Necturus* gallbladder epithelium is uphill, since the electrical potential across the membrane is ca. 65 mV, cell negative, and the intracellular Cl activity is ca. 36 mM, which, in normal amphibian Ringer (Cl activity ca. 86 mM) corresponds to $E_{Cl} = -23$ mV (Reuss & Weinman, 1979). Similar observations, i.e., intracellular Cl activity is higher than the activity predicted from equilibrium distribution, have been made in *Necturus* proximal tubule (Spring & Kimura, 1978), rabbit gallbladder (Duffey *et al.*, 1978), and small intes-

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tine (Armstrong *et al.*, 1979). In addition, in *Necturus* proximal tubule and in rabbit gallbladder it was found that the intracellular Cl activity decreased sharply after Na substitutions in the lumen (Spring & Kimura, 1978) or on both sides of the tissue (Duffey *et al.*, 1978).

Previous experiments in *Necturus* gallbladder have indicated that the luminal membrane is mainly K-permeable, but has measurable permeabilities for Cl and Na (Reuss & Finn, 1975*a, b*). The Na conductance of the luminal membrane is too low to account for Na entry on the basis of simple diffusion (Reuss & Finn, 1975*a, b*; Van Os & Slegers, 1975), even though the Na electrochemical potential difference is large (ca. 100 mV) and oriented in the direction that favors Na entry (Reuss & Weinman, 1979). A mechanism which would account for entry of NaCl into the cells in gallbladder and other leaky epithelia would be neutral uptake driven by the Na electrochemical potential difference (Spring & Kimura, 1978; Duffey *et al.*, 1978; Armstrong *et al.*, 1979; Reuss, 1979*a*; Reuss & Weinman, 1979).

Less information is available on the mechanism of NaCl transport across the basolateral membrane. Na transport is uphill (Reuss & Weinman, 1979), dependent on the activity of the Na-K ATPase located at the basolateral membrane (Reuss, Bello-Reuss & Grady, 1979; Van Os & Slegers, 1971). Cl transport, although downhill (Reuss & Weinman, 1979), cannot be explained by simple diffusion because of the low Cl conductance of the basolateral membrane (Reuss, 1979*c*). Cremaschi and Hénin (1975) found no radioactive Cl entry from the serosal solution into the cells, in rabbit gallbladder, under control conditions. Some labeling was observed, however, when the cells were depolarized.

The experiments reported in this paper were designed to study the effects of ionic substitutions in the bathing media on intracellular Cl activity. Cl substitutions with sulfate in the mucosal solution were employed to determine the time course of the changes of intracellular Cl. Unilateral and bilateral Na substitutions with several cations were employed to examine the role of luminal Na and the effect of cell depolarization on intracellular Cl. The results indicate that uphill Cl entry requires Na in the mucosal bathing medium, but net Cl entry also occurs during cell depolarization, when the Cl electrochemical potential difference across the cell membranes becomes favorable for Cl diffusion inward. It is suggested that Cl uptake across the basolateral membrane of depolarized cells involves an increase in Cl permeability.

Some of these results have been published in preliminary form (Reuss, 1979*a, b*).

Materials and Methods

Most of the methods have been described in detail previously (Reuss & Finn, 1975*a, b*, 1977; Reuss, 1978; Reuss & Weinman, 1979) and are summarized below.

Necturus gallbladders were mounted horizontally on a modified Ussing chamber which permits positioning of microelectrodes (ME) in the mucosal bathing solution. Impalements were performed with motorized remote control micromanipulators (Stoelting, Chicago, Ill.) under microscopic observation at 200× or greater, with a MS inverted microscope (Nikon Inc., Garden City, N.Y.) or a Biovert inverted microscope (Reichert, Austria). Both mucosal and serosal bathing media were exchanged continuously. NaCl-Ringer had the following composition (mM): NaCl, 109.2; KCl, 2.5; $NaHCO_3$, 2.4; $CaCl_2$, 1.0. Substitutions were isomolar when NaCl was replaced with other salts. Replacements of chloride with sulfate were isosmotic (sucrose addition). Care was taken to keep bathing solution K and Ca activities constant during exposure to Na_2SO_4 solutions (Reuss, 1979*c*).

Measurements with extracellular electrodes, intracellular conventional microelectrodes, and intracellular Cl-selective electrodes were performed as described before (Reuss & Weinman, 1979). Microelectrodes were prepared from inner-fiber glass capillaries (Frederick Haer and Co., Brunswick, Me., or W.P. Instruments, New Haven, Conn.). Conventional microelectrodes were filled with 3 M KCl or 4 M K acetate, and sometimes bevelled by the procedure of Ogden, Citron and Pierantoni (1978). The tip resistances were 20 MΩ or greater. Cl-selective ion-exchanger microelectrodes were prepared from the same glass. The tip was rendered hydrophobic by exposure to dimethyldichlorosilane or trimethylchlorosilane, followed by curing for 1 hr at 100 or 140 °C, respectively. The electrical connection between the resin and the electrometer was a reference KCl solution and a Ag-AgCl pellet or a chlorided silver wire inserted in the resin (Orme, 1969). The Cl exchangers employed were Corning 477315 (Corning Glass, Corning, N.Y.) or Orion 92-17-02 (Orion Research, Cambridge, Mass). The electrodes were calibrated at toom temperature (24 ± 1 °C) in single-salt solutions and in media similar in composition to the ones employed in the experiments. Slope and selectivity coefficients were determined as described by Fujimoto and Kubota (1976). Frequent checks of slope were done during the course of the experiment. The characteristics of the electrodes have been published previously (Reuss & Weinman, 1979). Intracellular Cl activity in tissues exposed to Cl-Ringer on the mucosal side was calculated from

$$a\mathrm{Cl}_i = a\mathrm{Cl}_o \exp \frac{(V_{\mathrm{Cl}} - V_{mc})\, zF}{nRT} \tag{1}$$

where aCl stands for Cl activity, the subscripts i and o refer to intracellular fluid and mucosal bathing medium, respectively, V_{Cl} is the change in potential measured by the Cl-selective electrode upon impalement, V_{mc} is the value of the apical membrane potential, n is a constant obtained from the calibration of each electrode, and the other symbols have their usual meaning. Since the bicarbonate concentration in the mucosal medium was low, bicarbonate contribution to the Cl electrode potential was neglected (*see* Reuss & Weinman, 1979). To calculate $a\mathrm{Cl}_i$ in tissues exposed to sulfate-Ringer, the term ($a\mathrm{Cl}_o$) in Eq. (1) was substituted by ($a\mathrm{Cl}_o + k\ a\mathrm{SO}_4^o$), where k is the selectivity coefficient of the Cl microelectrode (SO_4/Cl) and $a\mathrm{SO}_4^o$ is the sulfate activity in the bathing medium. The selectivity coefficient ranged from 0.15 to 0.21.

In each preparation, at least six impalements were performed with conventional microelectrodes and six with Cl-selective microelectrodes under control conditions. To study the effects of substitutions, the measurements were repeated at given intervals (several impalements); alternatively, both a conventional and an ion-selective microelectrode were kept in cells and their outputs measured continuously. The criteria for adequate impalement were those previously described (Reuss & Weinman, 1979; *see* Results).

To test the reliability of the measurement of aCl_i, impalements were performed in tissues exposed to sulfate-Ringer on both sides for 1 to 2 hours. Under these conditions, a mean "aCl_i" of 3.2 mM was measured. As shown previously by Spring and Kimura (1978) this probably represents the response of the microelectrode to other intracellular anions. This correction factor will be taken into account for the comparison of E_{Cl} and cell membrane potentials.

Conventional microelectrode output and transepithelial potential were measured with $10^{12}\,\Omega$ input impedance electrometers. Cl-selective microelectrode output was measured with a $10^{15}\,\Omega$ input impedance electrometer (model F 223A, W.P. Instruments, New Haven, Conn.). The outputs of the electrometers were amplified, displayed on the screen of an oscilloscope (Tektronix, Beaverton, Ore.), and either recorded on a three-channel pen recorder (Brush 2400, Gould Inc., St. Louis, Mo.) or digitized and stored in a signal averager (model 1074, Nicolet Instrument Corp., Madison, Wisc.). The stored data were read digitally and plotted (model 7010B X-Y recorder, Hewlett Packard, San Diego, Calif.).

Results are expressed as means ± SE. Statistical comparisons were done by conventional paired data analysis.

Results

As shown previously (Reuss & Weinman, 1979), impalements with Cl-selective microelectrodes under control conditions yield intracellular

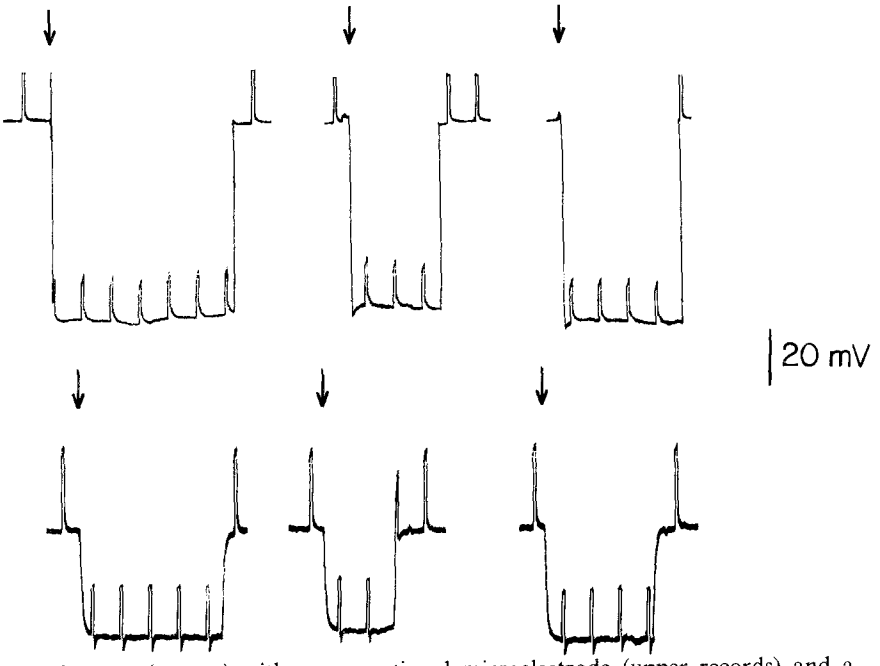

Fig. 1. Impalements (arrows) with a conventional microelectrode (upper records) and a Cl-selective microelectrode (lower records) across the apical membrane of different cells of the same preparation. Downward deflections upon impalement denote negative intracellular potential. Brief positive deflections in both records were produced by transepithelial current pulses, at 10-sec intervals, to measure the ratio of resistances of the cell membranes. Deflections in the conventional electrode in the extracellular position are off-scale. Note constancy of the values of potentials in different cells and negligible change in electrode tip potential upon withdrawal

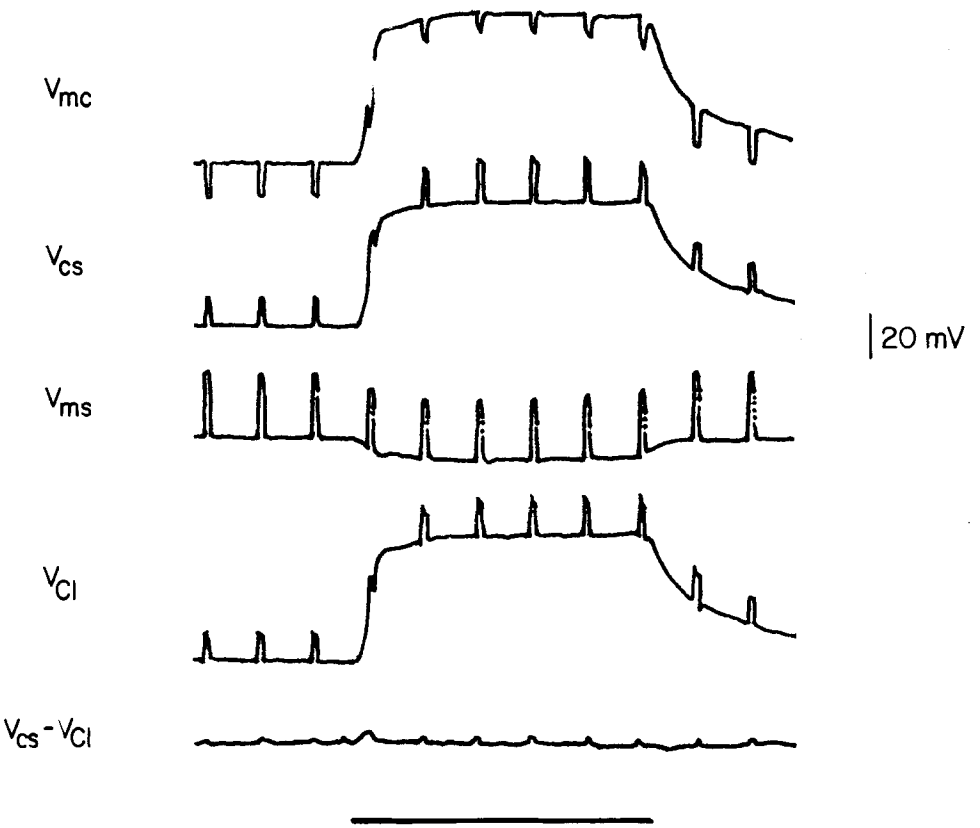

Fig. 2. Records of cell membrane potentials (V_{mc} and V_{cs}), Cl electrode potential (V_{Cl}), and transepithelial potential (V_{ms}) before, during, and after a complete substitution of Na with K in the mucosal side. Period of exposure to high-K medium is indicated by the line at the bottom of the figure. Records start with conventional and Cl-selective microelectrode in intracellular position. Upon exposure to K-Ringer, the changes of intracellular potential measured by the conventional and the Cl-selective electrode (V_{cs} and V_{Cl}, respectively) are essentially equal, as shown by the $V_{cs}-V_{Cl}$ trace. The large changes of intracellular potentials indicate high K selectivity of apical membrane of the impaled cells. The ratio of apical to basolateral membrane resistance (indicated by the voltage deflections in V_{mc} and V_{cs} records upon transepithelial current application) decreases during exposure to K-Ringer on the mucosal side because of the high K conductance of the apical membrane. Transepithelial current pulses were passed at 10-sec intervals. Record retraced to improve contrast

voltages negative to both bathing media, but less negative than the cell potential measured with conventional KCl or potassium acetate microelectrodes. Figure 1 shows typical impalements with a conventional and a Cl-selective microelectrode. The values obtained were usually stable, and quite similar in different cells in the same preparation (Reuss & Weinman, 1979). Figure 2 shows the effect of a brief substitution of K for Na on transepithelial and cell membrane potentials. During the

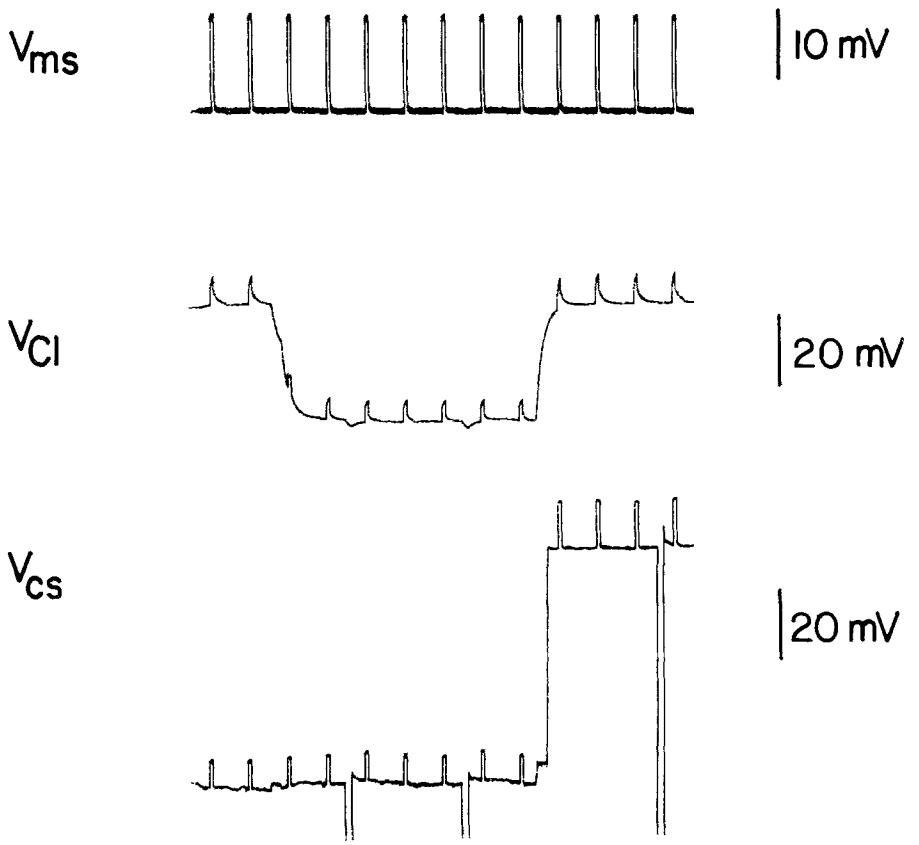

Fig. 3. Demonstration of electrical coupling between cells impaled with conventional electrode (V_{cs} trace) and Cl-selective electrode (V_{Cl} trace). Transepithelial potential is also shown (V_{ms}). Records start with conventional ME in a cell. After the second transepithelial pulse, a cell (ca. 80 μm away) was impaled with the Cl-selective microelectrode. Downward deflections in the V_{cs} record (off-scale) correspond to intracellular current pulses (5×10^{-9} A, 1 sec). Note that when both electrodes are inside the cells current application results in voltage change in the V_{Cl} record. After withdrawal, no voltage change is measured by the Cl-selective electrode when the same current pulse is passed through the conventional electrode. Transepithelial current pulses (upward deflections) were applied at 10-sec intervals

short time of this experiment the changes of intracellular potential measured by the conventional microelectrode and the Cl-selective microelectrode are large and essentially equal. In Figure 3, it is shown that two cells, one impaled with a conventional and one with a Cl-selective microelectrode, respectively, are electrically coupled. These observations strongly suggest that the impalements with both electrodes have not resulted in significant membrane damage, since the apical membranes of the two cells exhibit the same high K selectivity and the cells are electrically connected (*see* Discussion and Reuss & Weinman, 1979).

Effects of Removal of Cl from the Mucosal Medium on Intracellular Cl Activity

Table 1 summarizes the results of replacement of Cl in the mucosal medium with sulfate. The result is a predictable decrease of intracellular Cl activity (aCl_i). If the response of the Cl-selective microelectrode to other intracellular anions (3.2 mV) is taken into account, the steady-state

Table 1. Effect of mucosal Cl replacement with SO_4 on intracellular Cl activity

Condition	V_{cs} (mV)	aCl_i (mM)	aCl_i (corr) (mM)	aCl_{eq} (mM)
Control	-63.9 ± 1.6	34.0 ± 2.6	30.8 ± 2.6	7.0 ± 0.4
SO_4-Ringer	-61.2 ± 2.8	12.4 ± 1.5	9.2 ± 1.5	8.0 ± 0.8

$N=8$ experiments. Cl activity was measured before and 15–20 min after complete mucosal Cl substitution with SO_4 (sucrose added to isosmolality). V_{cs}: basolateral membrane potential; aCl_i: measured intracellular Cl activity; aCl_i (corr): corrected aCl_i (*see* Methods); aCl_{eq}: Cl activity predicted from equilibrium across the basolateral membrane. The fall of aCl_i is significant ($P<0.001$).

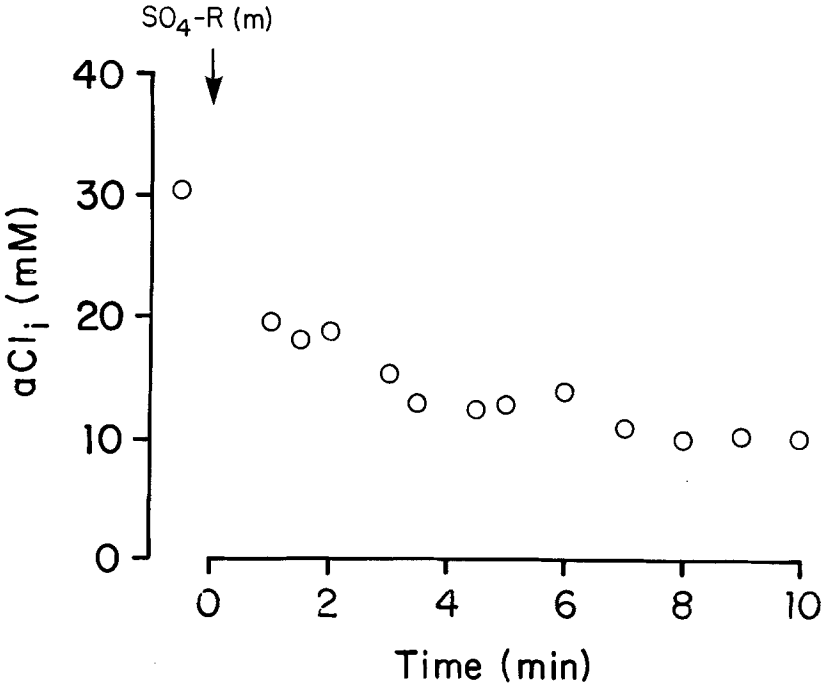

Fig. 4. Time course of decrease of intracellular Cl activity (aCl_i) in a tissue exposed to sulfate-Ringer on the mucosal side at the time indicated by the arrow. Control aCl_i value is mean of six impalements. Each experimental point was calculated from values obtained from one impalement with a conventional microelectrode and one impalement with a Cl-selective microelectrode. In this and similar experiments, a new steady-state aCl_i value was reached in less than 15 min

aCl_i values approach the equilibrium activity across the basolateral membrane. The time course of the reduction of aCl_i is illustrated in Fig. 4. A steady state in this and similar experiments was reached in about 10 min, in good agreement with the time course of cell volume reduction measured by Spring and Hope (1978, 1979). It is interesting to note that the decrease of aCl_i during exposure to sulfate-Ringer on the mucosal side (of 21.6 mM) is similar to our mean value of intracellular Na activity under control conditions, of ca. 20 mM (Reuss & Weinman, 1979). This would suggest that if all of the Cl loss from the cells takes place by NaCl extrusion aNa_i would decrease to zero. This is not possible. In addition, the recent measurements of Graf and Giebisch (1979), with a more selective Na electrode, yield a mean aNa_i in *Necturus* gallbladder close to 10 mM. Thus, only part of the net Cl efflux can be accounted for by NaCl extrusion, the remaining portion being probably KCl efflux (*see also* Weinstein & Stephenson, 1979).

Effects of Na Substitutions on Intracellular Cl Activity

In the experiments summarized in Table 2, several monovalent cations were employed to substitute Na in the mucosal medium. Lithium, choline, tetramethylammonium (TMA), and N-methyl-D-glucamine (NMDG) substitutions caused large reductions of aCl_i, which reached new steady-state values in 10 to 15 min. All of these effects were reversible, as illustrated by the two typical experiments shown in Fig. 5. Significant changes in cell potential took place under these conditions. Inasmuch as the serosal medium was always Na-Ringer, and since $P_{Na} > P_C$, where C is the replacement cation, a paracellular biionic potential oriented mucosa-positive resulted from these substitutions. In addition, mucosal Na substitutions change the equivalent electromotive force of the apical

Table 2. Effects of mucosal Na replacements on intracellular Cl activity

Replacement cation	aCl_i (control) (mM)	aCl_i (experimental) (mM)	P
NMDG	34.3 ± 4.0	10.5 ± 2.3	<0.025
Choline	29.9 ± 2.3	7.1 ± 0.5	<0.025
Li	30.3 ± 4.2	8.6 ± 0.5	<0.05
TMA	29.3 ± 3.8	7.8 ± 0.2	–

Number of experiments: 4 (NMDG), 3 (choline), 3 (Li), 2 (TMA). Timing of aCl_i measurements is as in Table 1.

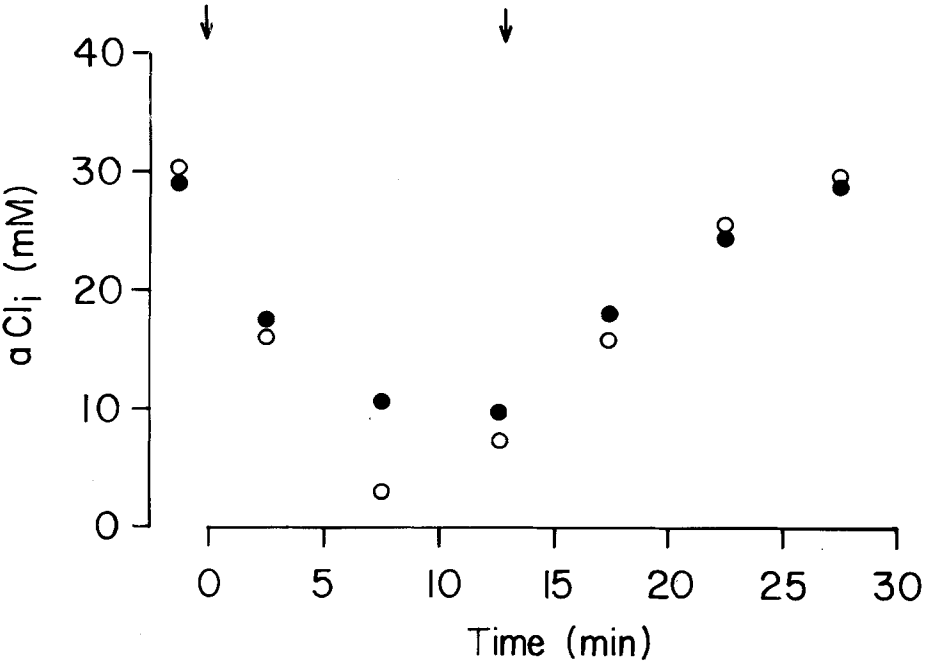

Fig. 5. Time course of changes of intracellular Cl activity (aCl_i) during mucosal Na substitutions with N-methyl-D-glucamine (NMDG, open circles) and tetramethylammonium (TMA, filled circles). Control values were calculated from means of six impalements. Values during Na substitutions were computed from at least three impalements in each 5-min period and plotted at the midpoint. First arrow indicates mucosal replacement of Na with NMDG or TMA. Second arrow indicates exposure to control NaCl-Ringer on the mucosal side. Throughout the experiment, the tissues were bathed with NaCl-Ringer on the serosal side

membrane, because the membrane has a small but finite Na permeability; the substitutions also produce an increase in the resistance of the paracellular pathway (Reuss & Finn, 1975*a*, *b*). The changes of cell membrane potential were mostly caused by the paracellular biionic potential and consisted of an increase of apical membrane potential (V_{mc}) and a decrease in basolateral membrane potential (V_{cs}). The mean increase of V_{mc} varied from 2.9 mV (TMA) to 16.1 mV (choline). The mean decrease of V_{cs} varied from 3.8 mV (Li) to 16.0 mV (TMA). Mean transepithelial potential changes were (all mucosa positive, in mV): Li, 8.0; TMA, 18.9; choline, 28.9; NMDG, 19.7. The final value of aCl_i in all these Na-free mucosal media was significantly decreased as compared to the control condition. As shown in Table 3, the aCl_i values in Na-free media (corrected for the nonspecific response of the microelectrode) were close to the activities predicted from equilibrium distribution across the luminal

Table 3. Comparison of corrected intracellular Cl activity and Cl activities predicted from equilibrium distribution across the cell membranes during exposure to mucosal Na-free media

Replacement cation	aCl_i (corr) (mM)	aCl_{eq} (mM)	
		Apical	Basolateral
NMDG	7.3±2.3	4.3±0.6	9.2±1.6
Choline	3.9±0.5	2.9±0.5	9.0±1.4
Li	5.4±0.5	3.8±0.4	5.3±0.6
TMA	4.6±0.2	4.4±0.6	9.1±1.9

Same experiments as in Table 2; aCl_i (corr) was calculated by subtraction of 3.2 mM from the aCl_i (experimental) values depicted in Table 2 (*see* Methods); aCl_{eq} was calculated from the cell membrane potential and the extracellular Cl activity. (*See* text.)

membrane. With the exception of the Li series, these aCl_i values seemed to be lower than the activities predicted from passive distribution across the basolateral membrane. Given the experimental errors involved in the measurements, a more quantitative analysis is not possible.

The changes of cell membrane potentials and intracellular Cl activity produced by Na substitutions were completely reversible, as illustrated in Fig. 5.

Bilateral Na substitutions with choline ($n=2$) or NMDG ($n=1$) had effects similar to those of the mucosal substitutions summarized in Table 2; aCl_i fell from 37.7±4.3 to 9.4±0.8 mM (values not corrected). When choline or NMDG were used to substitute for Na on the serosal side only, no significant change of aCl_i was observed (control: 30.0±0.3 mM; Na-free serosal: 27.8±1.1 mM).

Effects of Cell Membrane Potential on Intracellular Cl Activity

The results of replacements of mucosal Na with K were different from those described above. As shown in Table 4, exposure of the apical surface of the cells to a high-K, Na-free medium resulted in an increase of aCl_i. In this situation, because of the high K conductance of the apical membrane (Hénin & Cremaschi, 1975; Reuss & Finn, 1975*a, b*; Van Os & Slegers, 1975), the cell membranes depolarized immediately. This change in driving force will tend to move Cl into the cells. If the Cl conductance of the membrane(s) is high enough, net Cl entry can be explained by a passive, diffusional mechanism (*see* Discussion).

When Cl was omitted from the mucosal medium, cell depolarization produced by exposure to a high-K mucosal solution (K_2SO_4-Ringer) resulted in no change or in an increase of aCl_i (Fig. 6). Under these

Table 4. Effects of mucosal Na replacements with K on intracellular Cl activity

Condition	aCl_i (mM)	aCl_i (corr) (mM)	aCl_{eq}	
			Apical (mM)	Basolateral (mM)
Control	29.4±1.7	26.2±1.7	6.1±1.0	6.0±1.0
K-Ringer	43.7±3.2	40.5±3.2	71.4±3.3	40.4±2.4

$N=6$ experiments. Timing of measurements is as in the preceding tables. aCl_i increase in K-Ringer was significant ($P<0.02$). Corrected activities are compared with those calculated for equilibrium distribution across each cell membrane.

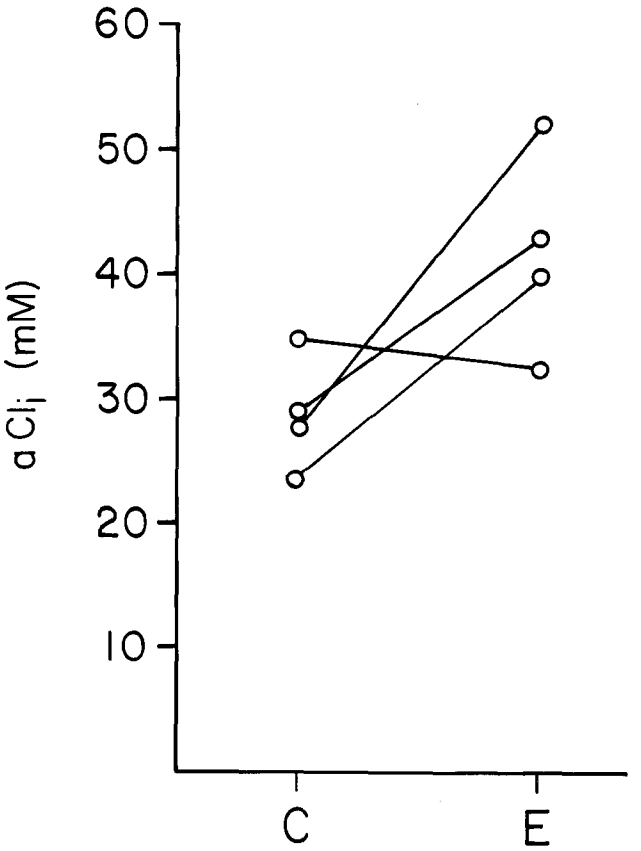

Fig. 6. Intracellular Cl activity (aCl_i) under control conditions (*C*) and 10–15 min after exposure to K_2SO_4-Ringer on the mucosal side (*E*). The serosal side was bathed with NaCl Ringer throughout the experiment. Lines join values calculated from at least four impalements with each microelectrode in an individual tissue

Table 5. Effects of bilateral Na substitutions on cell membrane potentials

Condition	V_{ms}	V_{mc}	V_{cs}
Control	-0.8 ± 0.1	-70.3 ± 1.0	-71.1 ± 0.9
Choline	-0.1 ± 0.4	-57.6 ± 2.9	-57.7 ± 2.7
P	NS	<0.005	<0.005
Control	-0.7 ± 0.2	-73.4 ± 1.1	-74.1 ± 1.0
NMDG	-0.1 ± 0.2	-72.6 ± 2.3	-72.3 ± 2.4
P	NS	NS	NS

Values in mV. $N=8$ experiments (choline), 6 experiments (NMDG). At least 6 cell potentials were measured before and at least 15 min after substituting all Na in both bathing media with choline or NMDG.

circumstances, the corrected value of $a\mathrm{Cl}_i$ (38.7 ± 4.1 mM) was not significantly different from the value predicted for equilibrium distribution across the basolateral membrane (45.2 ± 6.6 mM). Since Cl was not present in the mucosal solution, net Cl entry necessarily took place across the basolateral membrane.

Effects of Bilateral Na Substitutions on Cell Membrane Potential and Intracellular Cl Activity

Duffey *et al.* (1978) observed that substitution of choline for Na in mucosal and serosal media in rabbit gallbladder resulted in a decrease of $a\mathrm{Cl}_i$ and a mean reduction of cell membrane potential of about 10 mV. Both effects were reversible. They interpreted the depolarization as an indication of an electrogenic Na pump. In Table 5 we summarize the results of bilateral Na substitutions with choline or N-methyl-D-glucamine (NMDG) on cell membrane potentials and $a\mathrm{Cl}_i$ in *Necturus* gallbladder. Exposure to choline depolarized the cells by about 13 mV, but exposure to NMDG did not result in cell membrane potential changes, in agreement with previous results (Reuss & Finn, 1975*b*). This result suggests that cell depolarization in choline-Ringer is not caused by the absence of Na, but by the presence of choline.

Discussion

The measurements of cell membrane potentials and intracellular Cl activity in *Necturus* gallbladder epithelium have been recently subjected to strict validation criteria that have been described in detail (Reuss

& Weinman, 1979) and will be summarized here. These criteria include: (i) rapid change in microelectrode output upon impalement; (ii) stable intracellular record; (iii) equal tip potential values when the microelectrode is positioned in the bathing solution, before and after impalement; (iv) large ratio of cell membrane resistances (R_a/R_b); (v) electrical coupling of impaled cells; and (vi) large and nearly equal cell membrane potential change in two electrodes (conventional and Cl-selective), placed in two different cells, when the mucosal solution is substituted. When these criteria are satisfied, significant leaks or membrane damage are unlikely, and the measured values thus provide reliable estimates of the electrical properties of the membranes and the composition of the intracellular fluid.

Effect of Cl-Free Mucosal Medium on Intracellular Cl Activity

The intracellular Cl activity is about four times greater than the value predicted for electrochemical equilibrium. Omission of Cl from the mucosal bathing medium resulted in a decrease of aCl_i to a value close to equilibrium across the basolateral membrane in ca. 10 min. The time course of this effect is in good agreement with the estimation of Weinstein and Stephenson (1979) and the measurements of cell volume decrease in *Necturus* gallbladder during Na or NaCl substitutions on the mucosal side (Spring & Hope, 1978, 1979). It was important to establish this time course in order to decide the timing of measurement of aCl_i under other experimental conditions.

Effects of Na Substitutions on Intracellular Cl Activity

Na replacement with Li, NMDG, choline, or TMA on the mucosal side resulted in decreases of aCl_i to values close to those predicted from equilibrium distribution across the apical membrane in 10–15 min. Bilateral Na substitutions with NMDG or choline had the same effect as mucosal substitutions, whereas substitution of serosal Na alone resulted in no change of aCl_i. These observations prove that removal of Na from the apical surface of the cells is the cause of the reduction of aCl_i. Since both apical and basolateral membrane have finite Cl permeabilities (Reuss & Finn, 1975*b*; Reuss, 1979*c*) downhill net Cl efflux can take place at both cell borders. As appears to be the case during

Cl substitutions in the mucosal medium, the reduction of aCl_i during exposure to Na-free mucosal bathing solutions can result from net extrusion of both NaCl and KCl (Weinstein & Stephenson, 1979).

Mucosal Na replacement with K, on the contrary, resulted in an increase of aCl_i. Under these conditions, the Cl electrochemical potential difference reversed, becoming favorable for Cl entry, because the cells depolarized. Thus, net Cl entry could be explained by simple diffusion across the luminal membrane, the basolateral membrane, or both. Alternatively, luminal KCl neutral entry could account for the result. That this is not necessarily the mechanism involved was shown by the experiments summarized in Fig. 6. When the cells were depolarized by exposure to K_2SO_4-Ringer on the mucosal side aCl_i remained high or rose further, indicating that net Cl entry took place across the basolateral membrane. To examine the possibility of diffusional Cl entry across the luminal membrane of depolarized cells, aCl_i was measured (i) under control conditions and (ii) during exposure to Na-free media on the mucosal side and K_2SO_4-Ringer on the serosal side. Experiments with NMDG-Ringer on the mucosal side did not give conclusive results, because under these conditions the apical cell membrane potential is about 50 mV (cell negative), and aCl_i falls predictably. Exposure to KCl Ringer on the mucosal side and to K_2SO_4 on the serosal side resulted in an increase of aCl_i from 25.6 to 36.1 mM (means from two experiments, not corrected). This result shows that net Cl entry took place across the luminal membrane, but does not demonstrate that the mechanism is simple diffusion.

From these data, we conclude that cell depolarization produced by exposure to high external K results in Cl uptake by the epithelial cells. Substitution experiments indicate that Cl entry can take place across either membrane, down the electrochemical gradient. Uphill Cl accumulation was observed only in the presence of Na in the mucosal bathing medium.

Cremaschi and Hénin (1975) showed that tracer Cl did not enter the cells from the serosal side in rabbit gallbladder under control conditions; however, they observed significant tracer entry during cell depolarization. Our results agree with theirs. From ion substitution experiments on the serosal side we have calculated a basolateral membrane Cl transference number (t_{Cl}) of ca. 0.06 (Reuss, 1979*c*). During serosal exposure to Na-Ringer, the basolateral membrane resistance (R_b) is ca. 1,880 Ω cm^2. From these data and the Cl electrochemical potential difference across the membrane, the initial rate of Cl uptake by simple diffusion

after exposure to K_2SO_4-Ringer on the mucosal side can be calculated:

$$i_{Cl} = (E_{Cl} - V_{cs})\, g_{Cl} \tag{2}$$

where i_{Cl} is the inward Cl current, E_{Cl} is the Cl equilibrium potential ($=(RT/F)\ln(aCl_o/aCl_i)$, where aCl_o and aCl_i are the extra and intracellular Cl activities, respectively), and g_{Cl} is the Cl conductance ($=t_{Cl}/R_b$). Inserting $E_{Cl} = 22.4$ mV, $V_{cs} = 15$ mV, and $g_{Cl} = 3.2 \times 10^{-5}\ \Omega^{-1}\ cm^{-2}$, $i_{Cl} = 2.4 \times 10^{-7}\ A \times cm^{-2}$, or 2.5×10^{-12} moles $\times\ cm^{-2} \times sec^{-1}$. For a cell height of 20 μm, 1 cm^2 of epithelium contains 2×10^{-6} liter of intracellular fluid. Therefore, the initial maximum rate of increase of intracellular Cl concentration will be $2.5 \times 10^{-12}/2 \times 10^{-6}$ (moles $\times\ l^{-1} \times sec^{-1}$), or 0.08 meq $\times\ l^{-1} \times min^{-1}$. Even assuming no net water entry and no Cl efflux across the luminal membrane, this maximum possible rate (less than 2 meq/liter in 20 min) does not account for the elevation of aCl_i (of up to 17 meq/liter in 20 min, *see* Fig. 4). This calculation, and the tracer Cl experiments of Cremaschi and Hénin (*see* above) suggest that during cell depolarization basolateral membrane Cl permeability (P_{Cl}) increases. Since the effect is observed during exposure to K-Ringer on either side of the tissue, the increase of P_{Cl} seems to be voltage-dependent.

Effects of Bilateral Na Substitutions on Cell Membrane Potentials

As shown in Table 5, bilateral Na substitutions with choline resulted in significant cell depolarization. However, when NMDG was employed to replace Na the cell potential did not change significantly. Duffey *et al.* (1978) interpreted the cell depolarization observed during bilateral Na replacements with choline in rabbit gallbladder as an argument in favor of an electrogenic basolateral Na pump. Our results suggest that the effect is not due to the absence of Na in the bathing media, but to the presence of choline. Preliminary experiments, to be reported in detail elsewhere, indicate that choline enters the cells under these conditions (intracellular choline activity can be measured with K-selective electrodes, which have a higher selectivity for quaternary amines than for K). Choline entry has been demonstrated in squid axon (Hodgkin & Martin, 1965), red blood cells (Martin, 1977) and kidney slices of rat (Maizels & Remington, 1958), and *Necturus* (Whittembury, Sugino & Solomon, 1961). If choline entry results in net K efflux, the decrease of aK_i could account for the depolarization. The possibility of species differences, however, cannot be ruled out. Final resolution of this point will require further experimentation.

In conclusion, we have shown that Cl activity in the epithelial cells of *Necturus* gallbladder decreases sharply when mucosal Cl is removed, with a time course consistent with the change in cell volume detected by Spring and Hope (1978, 1979) under analogous experimental conditions. In addition, aCl_i decreases when Na is replaced with Li, NMDG, TMA or choline, on the mucosal side only or on both sides of the tissue. This observation supports the hypothesis of neutral NaCl entry at the luminal membrane of *Necturus* gallbladder, and is consistent with results in other leaky epithelia (Armstrong *et al.*, 1979; Duffey *et al.*, 1978; Spring & Kimura, 1978). Na replacements with K, however, cause increases of aCl_i, which can be explained by simple diffusion of Cl across the basolateral or both cell membranes. It is likely that cell depolarization results in an increase of basolateral Cl conductance. Decreases of aCl_i during exposure to both Cl-free or Na-free mucosal media appear to result from net cellular losses of both NaCl and KCl. Finally, bilateral Na substitutions result in cell membrane potential changes which depend on the substituting cation. Inasmuch as NMDG substitutions do not depolarize the cells, the depolarizing effect of choline substitution does not necessarily indicate the presence of an electrogenic Na pump. Of course, the lack of measurable depolarization during exposure to NMDG media does not prove that the Na pump is neutral.

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