J. Membrane Biol. 95, 229–240 (1987)

The Journal of
Membrane Biology

Intracellular Ion Concentrations in the Frog Cornea Epithelium During Stimulation and Inhibition of Cl Secretion

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Summary. The intracellular electrolyte concentrations in the isolated cornea of the American bullfrog were determined in thin freeze-dried cryosections using energy-dispersive X-ray microanalysis. Stimulation of Cl secretion by isoproterenol resulted in a significant increase in the intracellular Na concentration but did not change the intracellular Cl concentration. Similar results were obtained when Cl secretion was stimulated by the Ca ionophore A23187. Inhibition of Cl secretion by ouabain produced a large increase in the intracellular Na concentration and an equivalent fall in the K concentration. Again, no increase or decrease in the intracellular Cl concentration was detectable. Clamping of the transepithelial potential to ±50 mV resulted in parallel changes in the transepithelial current and intracellular Na concentration, but, with the exception of the outermost cell layer, in no changes of the Cl concentration. Only when Cl secretion was inhibited by bumetanide or furosemide, together with a decrease in the Na concentration, was a large fall in the Cl concentration observed. Application of loop diuretics also produced significant increases in the P concentration and dry weight, consistent with some shrinkage of the epithelial cells. The results suggest the existence of a potent regulatory mechanism which maintains a constant intracellular Cl concentration and, thereby, a constant epithelial cell volume. Through the operation of this system any variation in the apical Cl efflux is compensated for by an equal change in the rate of Cl uptake across the basolateral membrane. Cl uptake is sensitive to loop diuretics, directly coupled to an uptake of Na, and dependent on the Na and K concentration gradients across the basolateral membrane. Isoproterenol and A23187 seem to increase the Cl permeability of the apical membrane and thus stimulate Cl efflux. Ouabain inhibits Cl secretion by abolishing the driving Na concentration gradient for Cl uptake across the basolateral membrane.

Key Words intracellular electrolytes · epithelial transport · Cl secretion · cell volume regulation · frog cornea · isoproterenol · ionophore A23187 · ouabain · bumetanide · furosemide · X-ray microanalysis

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Introduction

Chloride secretion in many secretory epithelia is dependent on the availability of Na in the inner bath and can be inhibited by ouabain and loop diuretics. These and other findings led to a model of Cl secretion in which the transport of Cl is described as a secondary active process (Silva et al., 1977; Frizzell, Field & Schultz, 1979). Cl is taken up across the basolateral membrane of the epithelial cell by a cotransport with Na. In this way, the intracellular Cl concentration reaches values which are above electrochemical equilibrium allowing Cl to leave the epithelial cell passively across the apical membrane. Inhibition of Cl secretion by ouabain or Na-free inner bathing media can be explained by the reduction of the driving Na concentration gradient for the secondary active uptake of Cl, whereas loop diuretics are thought to inhibit the cotransporter directly.

Recently, we employed the technique of electron-microprobe analysis to measure the intracellular electrolyte concentrations in a Cl-secreting epithelium, the cornea epithelium of the frog (Rick et al., 1985). The behavior of the intracellular electrolyte concentrations during substitution of Na, Cl, and K in the outer or inner bath was found to be consistent with the above model. The results provided some evidence, however, that K is involved in the NaCl cotransport process and that the stoichiometry of the cotransporter might vary under certain experimental conditions. The values of the intracellular Na, K, and Cl concentrations were found to be in excellent agreement with data from ion-selective microelectrode measurements (Reuss et al., 1983).

In the present study we measured the electrolyte concentration in the frog cornea epithelium

during stimulation and inhibition of Cl secretion. The intracellular concentration of Cl was revealed to be surprisingly stable over a wide range of transepithelial transport rates. Only after direct inhibition of the cotransport system by furosemide or bumetanide was there a significant fall in the intracellular Cl concentration which was accompanied by a shrinkage of the epithelial cell volume. The results can be explained by a cellular control mechanism which keeps the rates of Cl uptake and efflux equal and, thereby, maintains a constant intracellular Cl concentration and cell volume.

Materials and Methods

Design and methods are identical to those employed in a previous study (Rick et al., 1985). In short, the experiments were performed on the cornea of the American bullfrog, *Rana catesbeiana* (West Jersey Biological Supply Farm, Wenonah, N.J.) After isolation, the pair of corneas were mounted on rings and inserted into Ussing-type chambers. Care was taken to avoid edge damage of the tissue. The half-chambers were continuously perfused with fresh bathing media which were pre-equilibrated with room air. Natural bulging of the cornea was maintained by applying a 1-cm H_2O hydrostatic pressure differential, inner (anterior chamber-side) bath positive. With the exception of one experimental series, the corneas were kept short-circuited throughout, using an automatic clamping device (custom made). The values for the short-circuit current (SCC) and transepithelial conductance (g_t) are calculated per nominal free cross-sectional area of the rings (38 mm^2) rather than true epithelial surface. The polarity of transepithelial potential values is inner minus outer bath.

Initially the corneas were incubated in Conway's or Ringer's solution until the SCC had reached a steady state. Cl secretion in one of the corneas was then stimulated or inhibited as described in Results. Usually the other cornea remained untreated, serving as a control. The composition of Conway's solution was (in mM): 67.6 NaCl, 25 $NaHCO_3$, 1.8 Na_2SO_4. 2.9 Na_2HPO_4, 1 Na-gluconate, 2.5 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$ and 26 glucose. The Ringer's solution contained: 110 NaCl, 2.5 $KHCO_3$, 1 $CaCl_2$, and 5 glucose. When equilibrated with air the solutions had a pH of 8.3 to 8.5 (Conway's) or 8.1 to 8.3 (Ringer's). Osmolarity was about 230 mOsm/liter. Isoproterenol, furosemide, and ouabain were obtained from Sigma (Munich). Bumetanide (a gift from Hoffmann-La Roche, Nutley, N.J.) and the ionophore A23187 (a gift from E. Lilly, Homburg) were applied from 1 mM stock solutions in ethanol and DMSO, respectively. The appropriate amounts of the solvent were added to the controls.

At the end of the experiments the rings were quickly removed from the incubation chambers. The epithelial surface was gently blotted with filter paper and covered with a thin layer of an albumin standard solution. For freezing, the corneas were plunged into a mixture of propane/isopentane kept at −196°C (Jehl et al., 1981). The time between removal of the rings from the chamber and freezing was between 5 and 15 sec. From the frozen tissue 1-μm-thick sections were cut at temperatures between −80 and −100°C in a modified Reichert cryoultramicrotome. The sections were mounted between two thin films (formvar or collodion) and subsequently freeze-dried at −80°C and 10^{-6} mbar. X-ray microanalysis of the freeze-dried sections was performed in a scanning electron microscope (Cambridge Stereoscan S150) which was equipped with an energy-dispersive X-ray-detecting system (LINK Systems). The acceleration voltage used was 20 kV, and the probe current selected was between 0.2 and 0.5 nA. Small areas (1 to 2 μm^2) within the cells or in the albumin standard layer were scanned for 100 sec and the emitted X-rays were analyzed in the energy range between 0 and 10 keV, which includes the K-lines of the biologically relevant light elements Na, Mg, P, Cl, K, and Ca. Discrimination between characteristic and noncharacteristic radiations (bremsstrahlung) was performed by a computer program (Bauer & Rick, 1978).

Quantification of the cellular element concentrations was achieved by comparing the intensities of the characteristic radiations obtained in the cells with those obtained in the adherent albumin standard layer. This method of quantification provides concentrations in units of mmol/kg wet weight. The cellular dry weight content was calculated from a comparison of the bremsstrahlung intensities of cell and standard and expressed as g% (g dry matter/100 g wet weight). Its value can be utilized to convert the wet weight concentrations into concentrations per liter cell water or kg dry matter. The standard solutions were prepared by dissolving 1 g purified bovine serum albumin (Behringwerke, Marburg) in 4 ml incubation medium. Further details of the preparation of freeze-dried cryosections for X-ray microanalysis and of the applied quantification method have been described earlier (Dörge et al., 1978; Rick, Dörge & Thurau, 1982).

Values given in the text are means ± SD with the number of observations in parentheses. In the Figures the intracellular concentrations are depicted as means ± SE, each point representing about 10 individual cellular measurements. In the Tables data from different experiments using the same protocol are combined. Differences which attained statistical significance in the pooled data generally attained significance already at the level of individual experiments. In all cases the Student's t-test for unpaired data was applied. The values for the relative stimulation or inhibition of the SCC were corrected for the spontaneous time course of the SCC observed in the untreated control cornea. The mean control SCC observed immediately prior to freezing was 34.5 ± 12.9 $\mu A/cm^2$ (n = 27).

Results

The values reported are mean intracellular concentrations obtained from separate measurements in the nucleus and cytoplasm or measurements in which the scanning area comprised about equal parts of the two cellular compartments. This format was chosen since, as already noted in the previous study (Rick et al., 1985), for small diffusible ions such as Na, Cl, and K no significant differences were detectable between nucleus and cytoplasm. This finding was confirmed for all experimental conditions employed in the present study. In contrast, for mainly structurally bound or compartmentalized elements such as Mg, Ca, and P, generally significant differences were observed. However, the relative distribution between the nucleus and cytoplasm remained unchanged under all experimental conditions justifying the presentation as mean intracellular values. The concentrations for Mg and Ca were

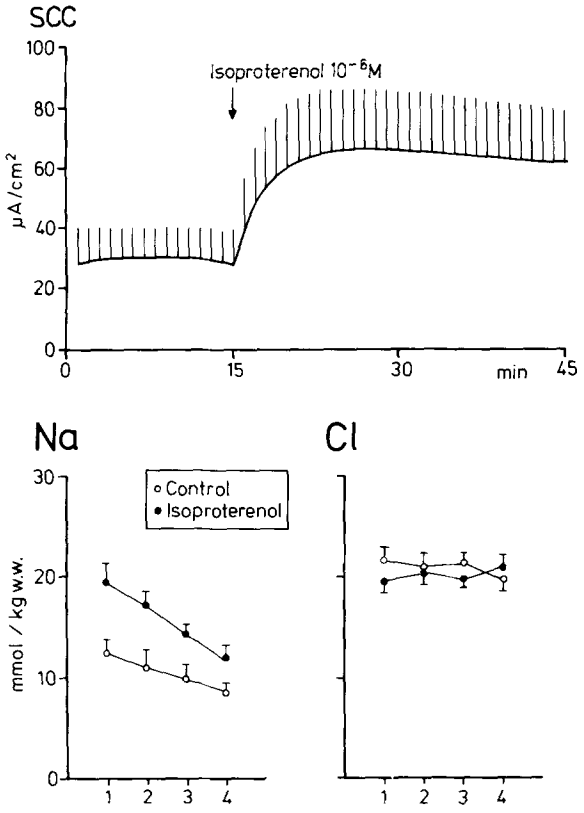

Fig. 1. Short-circuit current (SCC) and intracellular Na and Cl concentrations in the different cell layers of the cornea epithelium in control (Conway's) and after stimulation of Cl secretion by isoproterenol (30 min, drug added to the inner bath). The current deflections are caused by clamping the transepithelial potential to −10 mV for 500 msec, and represent a measure of the transepithelial conductance g_t. Cell layer 1 is the outermost, 4 the innermost cell layer of the epithelium. Means ± SE

always higher in the cytoplasm, whereas the P concentration was always higher in the nucleus. With the exceptions noted in the text, the concentrations of Mg and Ca were generally not significantly affected by the experimental perturbations applied and were similar to those reported previously (*see* Table 2 of Rick et al., 1985). Under several experimental conditions we observed parallel changes in the cellular dry weight and the P concentration. Since it is unlikely that the experimental perturbations change the total dry matter or P content of the cell, the variations in the dry weight and P concentration probably reflect cellular volume changes.

Throughout this study paired controls were used. This procedure was deemed necessary because of the relatively large scatter observed between individual animals (compare controls in Figs. 1 and 2). Corneas obtained from the same animal showed Na and Cl values which were within 1 mmol/kg wet wt when incubated under identical conditions. This finding was confirmed for control as well as isoproterenol-stimulated corneas.

Table 1. Effect of isoproterenol (10^{-6} M, about 30 min) on the intracellular Na, Cl, K and P concentrations and dry weight content (d.w.) of the cornea epithelium[a]

	Na	Cl	K	P	d.w.
	(mmol/kg wet weight)				(g%)
Control	8.3 ± 5.3	20.6 ± 6.9	120.7 ± 15.5	109.9 ± 23.5	22.8 ± 4.3
Isoproterenol	11.7[b] ± 7.1	20.6 ± 6.7	118.2[c] ± 17.2	108.3 ± 24.9	23.5 ± 6.1

[a] Mean ± SD of 372 (Control) and 417 (Isoproterenol) measurements obtained from 10 paired experiments. The bathing medium was Conway's solution.
Superscripts indicate the level of significance ([b] = $2P < 0.001$, [c] = $2P < 0.05$).

Stimulation of Cl Secretion by Isoproterenol

Figure 1 shows the typical response of the SCC and intracellular Na and Cl concentrations upon addition of the beta-agonist isoproterenol. The large stimulation of Cl secretion as evident from the SCC recording resulted in a significant increase in the intracellular Na concentration in all layers of the epithelium. The increase in the outer epithelial layers was generally more pronounced than in deeper layers, producing the impression of an increased inwardly directed Na gradient between the epithelial layers. In contrast, the Cl concentration in the various epithelial layers was not significantly altered. The stimulation of the SCC was accompanied by a similar rise in g_t as indicated by the increased height of the upstrokes in the SCC recording.

Table 1 summarizes the results obtained from 10 individual experiments. On the average, the SCC was stimulated by 89 ± 52%. In all but three of the experiments the increase in the Na concentration attained statistical significance. No clear-cut correlation was detectable between the degree of the Na increase and the relative stimulation of the SCC. However, in all three pairs of corneas with no detectable Na increase the SCC stimulation was below average. The K concentration showed a small decrease. In addition, we observed a small, statistically insignificant increase in the Ca concentration from 0.3 ± 0.9 to 0.5 ± 1.1 mmol/kg wet wt ($2P = 0.06$). In individual experiments, however, the change in the Ca concentration was significant.

In one further experiment, the effect of isopro-

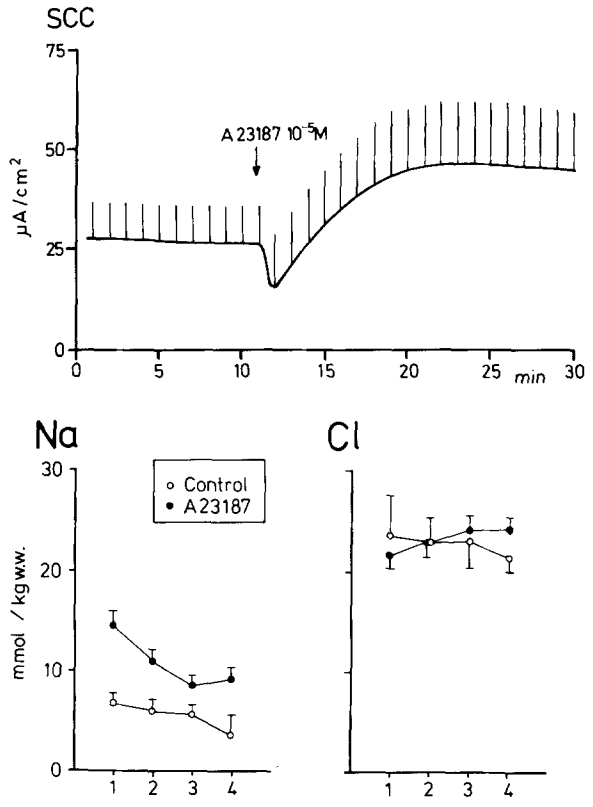

Fig. 2. Short-circuit current (SCC) and intracellular Na and Cl concentrations in the different cell layers of the cornea epithelium in control (Conway's) and after stimulation of Cl secretion by A23187 (19 min, drug added to the outer bath). For further explanations *see* legend to Fig. 1

terenol was tested in a pair of corneas incubated in Ringer's solution. The results were similar to those with Conway's solution illustrated in Fig. 1. The SCC was stimulated by 104% and the Na concentration increased from 9.0 ± 3.7 to 13.1 ± 4.3 mmol/kg wet wt ($n = 38$, $2P < 0.001$). Again, the Cl concentration remained constant at 26.2 ± 3.3 and 25.7 ± 4.0 mmol/kg wet wt, respectively. The higher Cl concentration in corneas incubated in Ringer's solution was noted earlier (Rick et al., 1985).

Stimulation of Cl Secretion by A23187

Figure 2 shows the effect of the Ca ionophore A23187 on the SCC and the intracellular Na and Cl concentrations in the cornea epithelium. Similar to isoproterenol, the ionophore produced a marked stimulation of the SCC and a significant increase in the intracellular Na concentration in all epithelial layers. The stimulation of the SCC was always preceded by a small transient inhibition. Table 2 summarizes the results obtained from three such experiments. The slight increase in the mean intracellular Cl concentration attained statistical significance in

Table 2. Effect of A23187 (10^{-5} M, about 20 min) on the intracellular Na, Cl, K, and P concentrations and dry weight content (d.w.) of the cornea epithelium[a]

	Na	Cl	K	P	d.w.
	(mmol/kg wet weight)				(g%)
Control	6.8 ± 4.0	19.3 ± 4.0	117.4 ± 14.3	105.3 ± 23.7	21.1 ± 2.2
A23187	11.2[b] ± 8.4	20.6[c] ± 4.4	112.2[c] ± 18.0	107.4 ± 22.5	20.7 ± 3.0

[a] Mean ± SD of 126 (Control) and 120 (A23187) measurements obtained from three paired experiments. The bathing medium was Conway's solution.
Superscripts indicate the level of significance ([b] = $2P < 0.001$, [c] = $2P < 0.05$).

only one pair of corneas. In contrast, the increase in the intracellular Na and the reciprocal change in the K concentration were detectable in all three experiments. On an average, the SCC was stimulated by 61 ± 24%. Surprisingly, the Ca concentration was identical under both experimental conditions (0.6 ± 0.5 mmol/kg wet wt).

Inhibition of Cl Secretion by Loop Diuretics

The effects of two different loop diuretics were investigated using two different experimental protocols. In experiments with the more potent inhibitor bumetanide, the drug was applied for 40 to 45 min to achieve an almost complete inhibition of the SCC. In contrast, furosemide was applied for only 10 to 15 min resulting in only a partial inhibition of Cl secretion. Furthermore, in all experiments with bumetanide the incubation medium was Ringer's solution, whereas in experiments with furosemide Conway's solution was used.

Figure 3 shows the typical effect of bumetanide on the SCC and the intracellular Na and Cl concentrations in the cornea epithelium. In addition to an almost complete inhibition of the SCC and a marked fall in g_t, a significant decrease in the intracellular Na and Cl concentrations is detectable in all epithelial layers. The intraepithelial Na gradient observed in the control cornea is almost completely abolished after bumetanide.

The effect of bumetanide was tested in nonstimulated corneas (Table 3) as well as in corneas which had been stimulated by isoproterenol (Table 4). In both groups the fall in the intracellular Na and Cl concentrations was highly significant. However, the relative as well as the absolute decrease of the Cl concentration was more pronounced in corneas stimulated by isoproterenol. Similarly, the inhibition of the SCC was at 93 ± 6% and 102 ± 5%, respectively, slightly higher in stimulated corneas. The decrease in the Na concentration was always

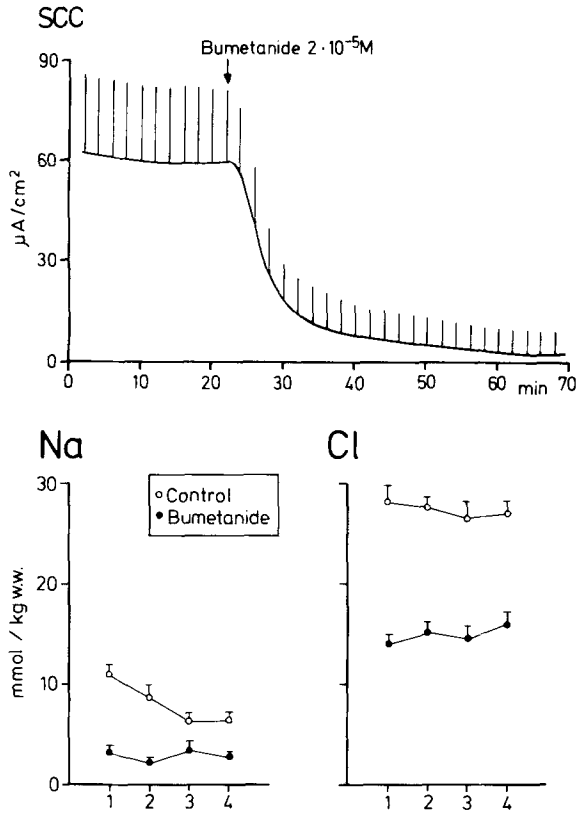

Fig. 3. Short-circuit current (SCC) and intracellular Na and Cl concentrations in the different cell layers of the cornea epithelium in control (Ringer's) and after inhibition of Cl secretion by bumetanide (46 min, drug added to the inner bath). For further explanations *see* legend to Fig. 1

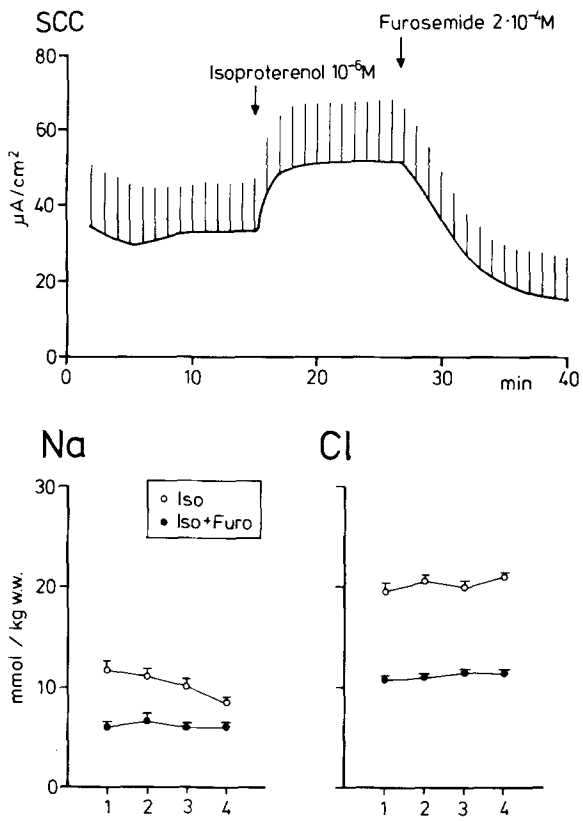

Fig. 4. Short-circuit current (SCC) and intracellular Na and Cl concentrations in the different cell layers of the cornea epithelium after stimulation of Cl secretion by isoproterenol (27 min) and subsequent additional inhibition by furosemide (14 min, drug added to the inner bath). For further explanations *see* legend to Fig. 1

Table 3. Effect of bumetanide (2×10^{-5} M, about 45 min) on the intracellular Na, Cl, K, and P concentrations and dry weight content (d.w.) of the cornea epithelium[a]

	Na	Cl	K	P	d.w.
	(mmol/kg wet weight)				(g%)
Control	7.3 ± 4.6	21.3 ± 5.0	117.1 ± 11.4	105.4 ± 20.8	21.2 ± 3.2
Bumetanide	2.5[b] ± 2.3	11.6[b] ± 4.3	127.3[b] ± 12.0	124.3[b] ± 18.5	24.6[b] ± 2.8

[a] Mean ± SD of 89 (Control) and 97 (Bumetanide) measurements obtained from three paired experiments. The bathing medium was Ringer's solution.
Superscripts indicate the level of significance ([b] = $2P < 0.001$).

Table 4. Effect of bumetanide (10^{-4} M, about 40 min) on the intracellular Na, Cl, K, and P concentrations and dry weight content (d.w.) of the cornea epithelium. Corneas were prestimulated by isoproterenol (10^{-6} M)[a]

	Na	Cl	K	P	d.w.
	(mmol/kg wet weight)				(g%)
Isoproterenol	9.9 ± 5.4	23.5 ± 5.8	118.2 ± 14.6	106.6 ± 19.7	21.8 ± 2.6
Isoproterenol + bumetanide	2.6[b] ± 2.3	9.6[b] ± 3.0	125.7[b] ± 13.3	130.3[b] ± 15.3	25.9[b] ± 2.4

[a] Mean ± SD of 87 (Isoproterenol) and 102 (Isoproterenol + bumetanide) measurements obtained from three paired experiments. The bathing medium was Ringer's solution.
Superscripts indicate the level of significance ([b] = $2P < 0.001$).

matched by an equivalent increase in the K concentration. In addition, increases in the P and dry weight concentrations were detectable, consistent with a reduction of the cell volume by between 15 and 20%. Bumetanide also induced a fall in the Ca concentration. However, only in stimulated corneas was this change statistically significant (from 0.4 ± 0.5 to 0.2 ± 0.3 mmol/kg wet wt, $2P < 0.02$). In addition, a small increase in the Mg concentration was observed. Combining both groups of corneas, the Mg value was increased from 8.6 ± 2.6 to 9.9 ± 2.8 mmol/kg wet wt ($n = 213$, $2P < 0.002$).

Very similar results were observed in experiments with furosemide. Figure 4 shows the typical

Table 5. Effect of furosemide (2×10^{-4} M, about 15 min) on the intracellular Na, Cl, K, and P concentrations and dry weight content (d.w.) of the cornea epithelium. Corneas were prestimulated by isoproterenol (10^{-6} M)[a]

	Na	Cl	K	P	d.w.
	(mmol/kg wet weight)				(g%)
Isoproterenol	10.3 ± 5.8	20.5 ± 5.3	120.9 ± 16.7	106.9 ± 22.3	22.4 ± 2.4
Isoproterenol + furosemide	6.2[b] ± 4.3	11.7[b] ± 3.7	120.5 ± 20.5	115.5[b] ± 31.8	23.9[b] ± 3.1

[a] Mean ± SD of 143 (Isoproterenol) and 155 (Isoproterenol + furosemide) measurements obtained from five paired experiments. The bathing medium was Conway's solution.
Superscripts indicate the level of significance ([b] = $2P < 0.001$, [c] = $2P < 0.02$).

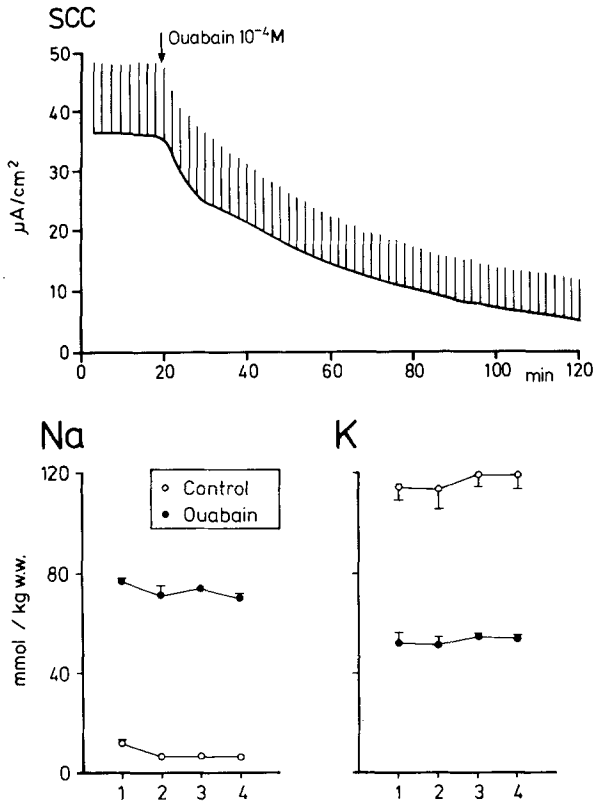

Fig. 5. Short-circuit current (SCC) and intracellular Na and K concentrations in the different cell layers of the cornea epithelium in control (Ringer's) and after inhibition of Cl secretion by ouabain (102 min, drug added to the inner bath). For further explanations *see* legend to Fig. 1

effect of furosemide in a cornea which first was stimulated by isoproterenol. Again, in all individual epithelial cell layers a highly significant fall in the Na and Cl concentration is detectable, despite the fact that the inhibition of the SCC is far from being complete. On an average of the five experiments summarized in Table 5, the SCC was inhibited by 57 ± 21%. Consistent with the lower degree of inhibition of Cl secretion the intracellular Na and Cl concentrations do not reach values as low as observed after bumetanide (*compare* Tables 4 and 5). Also, the increase in the P concentration and dry weight is less pronounced and no significant changes in the K, Ca, and Mg concentrations are detectable.

In one pair of corneas the effect of furosemide was tested without prior stimulation of Cl secretion by isoproterenol. Furosemide inhibited the SCC in this experiment by only 18% (15 min incubation time). The results were identical to those observed in stimulated corneas except for the fact that the Na concentration was not detectably changed and the fall in the Cl concentration was somewhat less pronounced (from 15.7 ± 3.6 to 10.0 ± 2.3 mmol/kg wet wt, $2P < 0.001$).

Table 6. Effect of ouabain (10^{-4} M, 102 min) on the intracellular Na, Cl, K, and P concentrations and dry weight content (d.w.) of the cornea epithelium[a]

	Na	Cl	K	P	d.w.
	(mmol/kg wet weight)				(g%)
Control	7.9 ± 3.9	21.6 ± 3.6	120.5 ± 14.5	103.2 ± 21.8	21.7 ± 4.9
Ouabain	71.9[b] ± 8.1	22.4 ± 4.2	53.1[b] ± 6.3	105.8 ± 21.5	22.4 ± 4.4

[a] Mean ± SD of 29 (Control) and 34 (Ouabain) measurements obtained from one paired experiment. The bathing medium was Ringer's solution.
Superscripts indicate the level of significance ([b] = $2P < 0.001$).

Inhibition of Cl Secretion by Ouabain

In three pairs of corneas Cl secretion was inhibited to various degrees by the cardiac glycoside ouabain. Figure 5 and Table 6 show the result of the experiment with the longest incubation time (102 min). The inhibition of the SCC (by 79%) was accompanied by a reduction in g_t, a large increase in the Na concentration, and an equivalent fall in the K concentration in all epithelial layers. Despite the drastic changes in the Na and K concentrations no significant change in the Cl concentration was detectable (Table 6). Also, the P concentration and dry weight were virtually unchanged, consistent with the notion that the epithelial cell volume is not affected.

Similar results were observed using shorter incubation times; however, the extent of the Na and K concentration changes were found to vary with the degree of transport inhibition by ouabain. 51 min after addition of ouabain the SCC was reduced

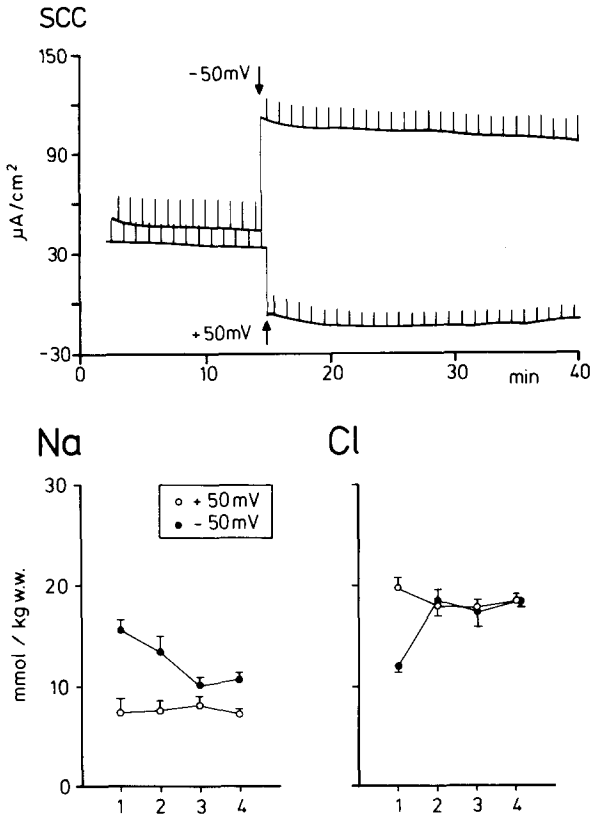

Fig. 6. Short-circuit current (SCC) and intracellular Na and Cl concentrations in the different cell layers of the cornea epithelium after clamping the transepithelial potential to +50 mV (25 min) or −50 mV (26 min). During voltage clamping the SCC actually reflects a clamp current. The open-circuit potentials of the corneas were +35 and +38 mV, respectively. The bathing solution was Conway's. Please note that the *y*-axis is offset by 30 mV. For further explanations *see* legend to Fig. 1

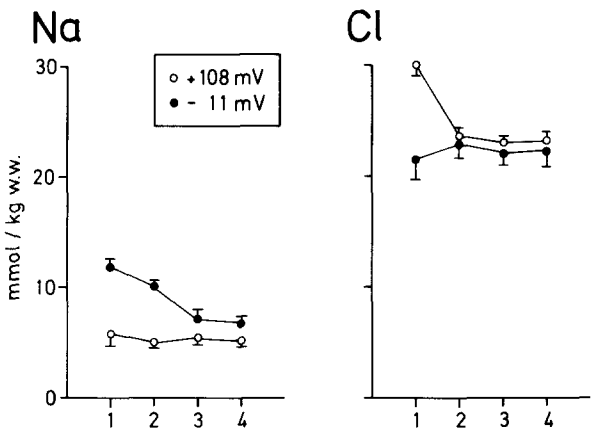

Fig. 7. Intracellular Na and Cl concentrations in the different cell layers of the cornea epithelium at transepithelial potentials of +108 and −11 mV, respectively. Hyperpolarization and depolarization was achieved by passing a constant current of ±30 μA/cm². The bathing solution was Ringer's. For further explanations *see* legend to Fig. 1

by 42% and the intracellular Na concentration was increased from 9.4 ± 3.7 (n = 40) to 49.8 ± 8.7 mmol/kg wet wt (n = 37, $2P < 0.001$). After 28 min of ouabain the inhibition of the SCC amounted to 32% and the Na concentration increased from 10.1 ± 3.9 (n = 38) to 36.7 ± 6.2 mmol/kg wet wt (n = 41, $2P < 0.001$). The increase in the Na concentration was in all cases matched by a reciprocal fall in the K concentration so that the sum of intracellular Na and K remained constant. On an average, the Ca concentration was not detectably changed. However, in one experiment (51-min ouabain) the Ca concentration increased from 0.3 ± 0.6 to 0.9 ± 1.6 mmol/kg wet wt, a change which almost reached statistical significance ($2P$ = 0.06).

Effect of Transepithelial Voltage

In a further experimental series Cl secretion was stimulated or inhibited by varying the transepithelial electrical potential. Two different protocols were applied. In three experiments the transepithelial potential was clamped to nonzero potentials (± 50 mV) while in a fourth experiment a current clamp rather than a voltage clamp was used (± 30 μA/cm²).

As shown in Fig. 6, clamping of the potential to −50 mV resulted in more than a doubling of the transepithelial current, whereas clamping to +50 mV resulted in a small, negative current. Under both conditions was a reduction in g_t detectable. At −50 mV the Na concentration in all layers was significantly increased and, similar to isoproterenol and A23187, a marked intraepithelial Na concentration gradient was apparent. In contrast, at +50 mV the intraepithelial Na gradient was completely abolished. The Cl concentration remained essentially unchanged except for the outermost cell layer. In the experiment shown in Fig. 6 a large and highly significant fall in the Cl concentration of the first cell layer was observed at −50 mV. In other experiments the fall in the Cl concentration at −50 mV was less pronounced and, instead, a slight increase in the Cl concentration of the outermost layer was detectable at +50 mV.

The current clamp used in the fourth experiment (±30 μA/cm²) resulted in a transepithelial potential difference at the time of freezing of +108 mV and −11 mV, respectively. The results were similar to the voltage-clamp experiments, except for the fact that at −11 mV the Cl concentration of the first cell layer was not detectably different from deeper layers while at +108 mV a large increase in the Cl concentration of the first cell layer became apparent (*see* Fig. 7).

The mean Na concentration in hyperpolarized (+50 and +108 mV) and depolarized corneas (−50 and −11 mV) was 6.0 ± 3.6 (n = 157) and 9.6 ± 5.6 mmol/kg wet wt (n = 161, $2P < 0.001$), respectively. These values were slightly lower and higher, respectively, than mean values observed in short-circuited corneas (controls). The fall in the Cl concentration in the first cell layer was always accompanied by significant increases in the P and dry weight concentrations, indicating some cellular shrinkage. Conversely, increases in the Cl concentration of the first layer were accompanied by a fall in P and dry weight, consistent with cellular swelling.

SCC and Transepithelial Conductance

As evident from the Figures, changes in the SCC were generally paralleled by changes in g_t. The only notable exception was depolarization of the transepithelial potential to −50 mV which resulted in a fall of g_t despite a large increase in the current. Isoproterenol resulted in an increase of g_t from 0.99 ± 0.31 to 1.82 ± 0.57 mS/cm^2 (n = 11). Based on an average current increase of 27.3 ± 18.1 μA/cm^2 the apparent transepithelial driving force of Cl secretion E_{Cl} amounts to 33 mV. This value is slightly lower than the value of 37 mV previously obtained in control corneas (Rick et al., 1985). After bumetanide g_t averaged 0.47 ± 0.12 mS/cm^2 (n = 6), a value almost identical to the apparently SCC-unrelated component of g_t calculated from the relationship between g_t and SCC (Rick et al., 1985). For interpretation of the present g_t data it should be noted, however, that the g_t values in the frog cornea are critically dependent on the applied transepithelial hydrostatic pressure difference. In preliminary experiments we observed a significant g_t decrease when the transcorneal hydrostatic pressure difference was increased from 1 to 10 cm H_2O, anterior chamber-side positive. The control as well as the isoproterenol-stimulated SCC were unaffected by this maneuver suggesting that the effect is on the paracellular rather than transcellular transport pathway.

Discussion

Regulation of Intracellular Cl Concentration

A rather unexpected finding of the present study is the fact that stimulators or inhibitors of Cl secretion, such as isoproterenol, A23187, or ouabain, did not change significantly the intracellular Cl concentration. Based on a transcellular transport model, this result suggests that Cl uptake and Cl extrusion in the cornea epithelium are equally stimulated or inhibited. Considering their pharmacological diversity, however, it seems rather unlikely that all these drugs exhibit a dual, equal action on each of the two transport barriers. More likely, the result can be explained by the presence of an intracellular control mechanism that very effectively synchronizes the rate of Cl uptake and extrusion. Similar to our results, Welsh and Ruppert (1983) observed in the canine tracheal epithelium no change or actually a slight increase in the Cl activity after stimulation by epinephrine. However, other authors have reported a significant decrease using the same preparation (Shorofsky, Field & Fozzard, 1984).

Obviously, intracellular Ca and pH may play a role in synchronizing the Cl transport across the apical and basolateral cell membranes. Ca directly stimulates the apical Cl channel (Frizzell, Rechkemmer & Shoemaker, 1986) as well as the basolateral K channel (Welsh & McCann, 1985), and might also have an effect on furosemide-sensitive cotransport systems (Lauf, 1985). It is conceivable that changes in the Na concentration associated with stimulation or inhibition of Cl secretion (*see* Figs. 1–4) are translated into Ca activity changes via a Na/Ca exchange across the basolateral membrane. A similar case could be made for the intracellular pH (for discussion *see* Rick et al., 1985).

With regard to a possible role for intracellular Ca, the present investigation provides some evidence. In several of the experiments with isoproterenol and ouabain a significant increase in the Ca concentration was observed. Conversely, after bumetanide a decrease in the Ca concentration was detectable. This behavior of the intracellular Ca concentration is consistent with a Na/Ca countertransport at the basolateral membrane. A Na/Ca exchange could also account for the rise of intracellular Ca seen after removal of Na from the inner bath (Rick et al., 1985). In interpreting these findings it should be kept in mind, however, that electron microprobe analysis provides only total element concentrations. Therefore, it is entirely possible that the intracellular Ca activity is unchanged. Furthermore, due to the limited spatial resolution power of analysis, the possibility cannot be excluded that the increase or decrease in intracellular Ca is confined to a subcellular compartment like the smooth endoplasmic reticulum and, thus, not visible to the cell membrane. It should also be noted that energy dispersive X-ray microanalysis, as applied in this study, is not very sensitive for the detection of intracellular Ca, mainly because of an overlap of the characteristic X-ray lines of K and Ca. This might explain why we were unable to de-

Table 7. Effect of bumetanide (2×10^{-5} M) on the intracellular contents of Na, K, Cl and water (H_2O) in the cornea epithelium[a]

	Na	K	Cl	H_2O
	(mmol/kg d.w.)			(liter/kg d.w.)
Control	34	549	99	3.69
Bumetanide	10	517	47	3.06
Δ	−24	−32	−52	−0.62
Isoproterenol	45	542	107	3.59
Isoproterenol + bumetanide	10	485	37	2.86
Δ	−35	−57	−70	−0.73

[a] Data are from Tables 3 and 4, respectively. Element contents are calculated by multiplying the wet weight concentration with 100/d.w. The water content is evaluated as (100-d.w.)/d.w.

Table 8. Intracellular concentrations of Na, K, Cl in the cornea epithelium per liter cell water[a]

	Na	K	Cl	Σ ions
	(mmol/liter cell water)			
Control	10.7	156.3	26.7	193.7
Isoproterenol	15.3	154.5	26.9	196.7
Control	9.3	148.6	27.0	184.9
Bumetanide	3.3	168.8	15.4	187.5
Control	10.1	153.9	27.6	191.6
Ouabain	92.7	68.5	28.8	190.0

[a] Data are from Tables 1, 3, and 6. Concentrations are calculated by multiplying the wet weight concentration with 100/(100-d.w.).

tect any significant change in the intracellular Ca concentration after addition of the Ca ionophore A23187.

Epithelial Cell Volume and Cl Secretion

As discussed previously (Rick et al., 1985), Cl secretion is the result of two processes directly involved in the regulation of the epithelial cell volume. The active process, presumably a coupled uptake of Na, K and 2Cl across the basolateral cell membrane, leads to a gain of intracellular solutes and, therefore, should increase cell volume. Conversely, the passive process, an electrically coupled efflux of Cl and K across the two opposing cell membranes, leads to a loss of intracellular solutes and, consequently, should decrease cell volume. Thus, the maintenance of a constant intracellular Cl concentration during stimulation and inhibition of Cl secretion may be viewed as a byproduct of a very effective epithelial cell volume regulation. Indeed, under all conditions in which the Cl concentration remained constant, our measurements of the dry weight and P concentration indicated an unchanged epithelial cell volume. Vice versa, the fall in intracellular Cl observed after furosemide or bumetanide was always accompanied by a rise in the P concentration and dry weight, indicating some cellular shrinkage.

Table 7 lists the intracellular contents of the three diffusible ions Na, K, and Cl before and after addition of bumetanide, the experimental procedure which produced the largest reductions in the cell volume as judged by the change in the P and dry weight concentrations. It is evident that in nonstimulated as well as in stimulated corneas the cell shrinkage is accompanied by an efflux of Na, K, and Cl. The loss of Cl is lower than combined losses of Na and K suggesting that another anion is lost, perhaps bicarbonate. In stimulated corneas the efflux of all three ions is significantly higher compared to nonstimulated corneas. In part, this may be explained by an increased permeability of the cell membrane to K and Cl in the presence of isoproterenol.

The fluid lost by the cells appears to be isotonic. Dividing the combined losses of the Na, K, and Cl by the change in the water content, the salt concentration of the extruded fluid can be calculated. For nonstimulated and stimulated corneas this value amounts to 174 and 221 mmol/liter, respectively, values which are only slightly lower than the total salt concentration of the Ringer's solution (228 mmol/liter). Based on the cellular dry weight, also the ion concentrations in the intracellular water space can be calculated. Table 8 lists these values for Na, K, and Cl under three of the experimental conditions tested. The sum of concentrations of the three ions is surprisingly constant between different experimental series and is not detectably influenced by isoproterenol, bumetanide or ouabain. Again, the values are only slightly lower than the salt concentration of the Ringer's solution consistent with the view that the three ions account for most of the intracellular osmolality. It is further evident that the recalculation of the data into water concentration does not change the thrust of the results. To the contrary, some effects seem to be emphasized in this way, for example, the K increase after bumetanide.

Cl Uptake Across the Basolateral Membrane

The parallel fall in the intracellular Na and Cl concentration after furosemide or bumetanide is consis-

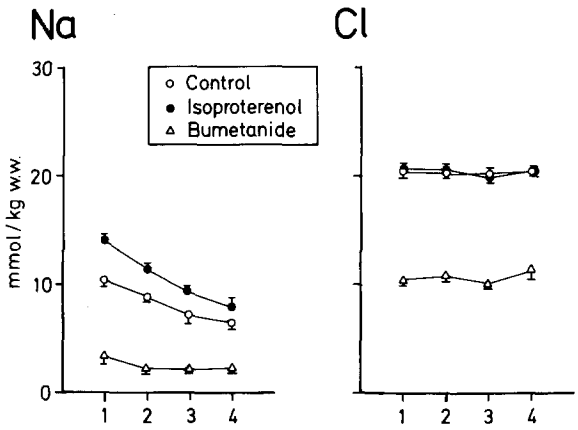

Fig. 8. Intracellular Na and Cl concentrations in the different cell layers of the cornea epithelium in control, after isoproterenol, and after bumetanide. The bumetanide data are combined from stimulated and nonstimulated corneas. Error bars were calculated from the SE's observed in the individual experiments following the rules of error propagation. For further details *see* legend to Fig. 1

tent with the view that Cl uptake is mediated by a NaCl cotransport system at the basolateral membrane. A similar, though slightly less pronounced fall in the intracellular Cl activity has been observed in microelectrode studies (Patarca, Candia & Reinach, 1983). The inhibition of the Cl efflux across the apical membrane can be explained as a secondary effect due to the fall in the intracellular Cl concentration. The extremely low values for the intracellular Na concentration after bumetanide suggest that the cotransporter is the only appreciable source of Na influx in the cornea epithelium. The values are even lower than those observed after removal of Na from the inner bath (Rick et al., 1985), a seeming discrepancy which may be explained by the longer incubation times used in the present study.

Further evidence for a direct coupling between influx of Na and Cl across the basolateral membrane is the increase in the Na concentration after stimulation of Cl secretion by isoproterenol or A23187. The same positive correlation between the rate of Cl transport and the intracellular Na concentration has been observed under a variety of experimental conditions (Rick et al., 1985). An inverse relationship between the intracellular Na concentration and the rate of Cl transport should prevail if the primary variable were the intracellular Na concentration rather than the rate of NaCl cotransport. This situation seems to be realized when the Na pump is inhibited by ouabain or by removal of extracellular K.

In an earlier investigation we observed a significant fall in the intracellular Cl concentration and Cl secretion upon removal of K from the inner bath (Rick et al., 1985). This result suggested to us a direct involvement of K in the Cl uptake across the basolateral membrane. However, at the same time we noted an increase in the intracellular Na concentration, raising the possibility that the inhibition of Cl uptake is caused by the reduced Na gradient. In the present experiments with ouabain we obtained much larger increases in the intracellular Na concentration without any change in the intracellular Cl concentration. We conclude, therefore, that the increase in the Na concentration *per se* is not sufficient to account for a fall in Cl, and that the decrease in Cl after K removal points to the involvement of K in the Cl uptake mechanism. The K dependence of the Cl uptake could be explained by a Na, K, 2Cl carrier system as described in erythrocytes (Dunham, Stewart & Ellory, 1980) and the rectal gland of the dogfish (Greger & Schlatter, 1984).

The changes in the intracellular ion concentrations after ouabain are consistent with the view that the inhibition of Cl secretion by ouabain is due to a reduction of the driving Na gradient for the Cl uptake. In fact, with increasing inhibition of the SCC the combined chemical driving force for a putative Na, K, 2Cl carrier at the basolateral membrane seems to approach zero. In the experiment with the longest incubation time (data from Table 6) the driving force is actually slightly reversed (−10 mV), favoring Cl extrusion rather than Cl uptake. However, it is entirely possible that some of the assumptions made in this calculation are incorrect. If the K concentration in the lateral spaces of the epithelium were only slightly higher than in the inner bath (3.7 rather than 2.5 mM), or the activity coefficient of the intracellular ions were only slightly lower than assumed (0.70 rather than 0.76) the actual gradient would be zero. It is conceivable also that the stoichiometry of the cotransporter in the cornea is different from that observed in other tissues or that, as discussed previously (Rick et al., 1985), the stoichiometry is variable.

In controls the calculated driving force for the Na, K, 2Cl carrier varied between 25 and 29 mV. Isoproterenol and A23187 reduced this value by about 10 mV. In contrast, after bumetanide the gradient increased to 85 mV. The inverse relationship between driving force and transport rate suggests that the cotransport system is stimulated by isoproterenol and inhibited by bumetanide.

Cl Efflux Across the Apical Membrane

It appears that intracellular modulators of Cl secretion such as cAMP and Ca directly stimulate the

apical Cl channel (Greger, Schlatter & Gögelein, 1985; Frizzell et al., 1986). The present results with isoproterenol and A23187 are consistent with this view. Since the chemical gradient is unchanged and the electrical driving force, at least after epinephrine (Nagel & Reinach, 1980), is not significantly changed, the increased apical Cl efflux can only be accounted for by an increased Cl permeability. The present investigation does, however, not directly answer the question of whether the effect on the apical Cl permeability is the primary event in the stimulation of Cl secretion, as seems to be the case in the dog trachea (Welsh & Ruppert, 1983) and dogfish rectal gland (Greger et al., 1984). To further elucidate the temporal sequence of the events involved in the stimulation of Cl secretion in the frog cornea, time course studies will be needed.

Epithelial Cooperation in Cl Secretion

Stimulation and inhibition of Cl secretion were accompanied by parallel changes in the height of an intraepithelial Na concentration gradient (*see* Figs. 1–4). In contrast, the intraepithelial concentration profile for Cl was generally flat. This is clearly demonstrated by Fig. 8 which shows the average Na and Cl concentrations in the different epithelial layers under three different conditions: in control, after stimulation of Cl secretion by isoproterenol, and after inhibition of Cl secretion by bumetanide. The Na concentration gradient between outer and inner epithelial layers can be explained by two possible models of epithelial cooperation (Rick et al., 1985). The *pump gradient* hypothesis assumes that the influx of NaCl is the same in all epithelial layers and that the cells of the outer layers have a lower capacity to extrude Na. Conversely, the *influx gradient* hypothesis assumes that the Na pump capacity is the same in all layers and that the NaCl influx is mainly confined to the outermost layer. Both models result in a Na concentration gradient and consequently Na diffusion from the outer to the inner epithelial layers. In either case, the Na gradient should vary with the rate of NaCl influx and, therefore, with the rate of transepithelial Cl secretion. However, even after completely abolishing transepithelial Cl transport with bumetanide the Na concentration in the outermost cell layer remains slightly elevated, suggesting that there is a small, bumetanide-insensitive Na leak.

With the exception of the voltage-clamp experiments no intraepithelial Cl concentration gradient was detectable. At first glance, this finding seems to favor the influx gradient model, since if all Cl influx is into the outermost cell layer there is no need for a concentration gradient to drive Cl from the deeper to the outer layers. However, as pointed out earlier (Rick et al., 1985), in the pump gradient model a small intraepithelial electrical potential gradient—the cell membrane potential in the deeper layers being slightly more negative than the outer layers—might account for Cl diffusion between the layers. The presence of the Cl gradient only in the voltage-clamp experiments may be explained by the wide range of transepithelial voltages applied, resulting in larger current changes than under any other experimental conditions. In part, however, a reduced epithelial cooperation may have played a role. It is well known that membrane depolarization leads to uncoupling of the cell-to-cell channel (Obaid, Socolar & Rose, 1983). In effect, uncoupling of the transport syncytium would reduce the available basolateral membrane area and, therefore, would reduce the ability of the cotransport system to compensate for primary changes in the Cl efflux. The observed fall and rise in the Cl concentration is, of course, consistent with a primary effect of voltage clamping on the Cl efflux. Transepithelial depolarization should increase the apical membrane potential and, therefore, increase apical Cl efflux, while transepithelial hyperpolarization should have the opposite effect. Shrinkage and swelling of the first cell layer under these conditions suggest that voltage clamping also changes the K efflux from the first cell layer, either across the basolateral membrane or via the cell-to-cell junctions into deeper layers of the epithelium.

The authors wish to thank Brigitte Mayer, Birgit Sauer, Gertrud Spancken, Elborg von Arnim-Spellenberg, Erwin Strigl, and Monika Weigel for their excellent technical assistance. We would also like to thank Drs. Peter Reinach and Eberhard Schlatter for critically reviewing this manuscript. Financial support was provided by grants from the Deutsche Forschungsgemeinschaft.

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Received 23 June 1986