J. Membrane Biol. 142, 9–20 (1994)

The Journal of
Membrane
Biology

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Cell Cl and Transepithelial Na Transport in Toad Urinary Bladder

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Received: 8 October 1992/Revised: 22 July 1994

Abstract. Relationships between short-circuit current (I_{sc}), cell Cl and the mechanism(s) of Cl accumulation in toad bladder epithelial cells were investigated. In serosal Cl-free gluconate Ringer, 80% of the cell Cl (measured by x-ray microanalysis) was lost over 30–60 min with an associated decrease in cell water content. Concomitantly, I_{sc} fell to 20% of its initial value within 10 min but then recovered to 45% of its initial value despite continued Cl loss. With the reintroduction of Cl, cell Cl and I_{sc} both recovered within 10 min. Serosal SITS (4-acetamido-4′-isothiocyano-stilbene-2,2′-disulfonate; 0.5 mM) plus bumetanide (0.1 mM), did not prevent the fall in I_{sc} or the loss of cell Cl in gluconate medium, although they did inhibit subsequent recovery of I_{sc} in this medium. They also prevented the recovery of I_{sc} in Cl medium but not the reaccumulation of Cl by the cells. Although SITS and bumetanide did not prevent the loss or recovery of Cl, they modified the pattern of the ion changes. In their absence, changes in cellular Cl were twice that of the changes in measured cellular cations implicating basolateral Cl/HCO_3 exchange in Cl movement. With SITS plus bumetanide present, changes of similar magnitude in Cl were associated with equivalent changes in cation, consistent with the inhibition of Cl/HCO_3 exchange.

Key words: Cell Cl — Cl/HCO_3 exchanger — Toad bladder — X-ray microanalysis — Cell volume

Introduction

The nature of the medium anion has important effects on Na transport by tight absorbing epithelia such as toad urinary bladder (Singer & Civan, 1971). For example, replacement of either mucosal or serosal Cl with the impermeant anion gluconate inhibits Na transport (Nielsen, 1984; Lewis et al., 1985). In the toad bladder, mucosal gluconate is thought to act by reducing the apical Na permeability (Lewis et al., 1985). In contrast, in both toad bladder (Lewis et al., 1985) and frog skin (Leibowich, DeLong & Civan, 1988), the inhibition of Na transport with serosal gluconate appears to occur through a volume-sensitive reduction in basolateral membrane K conductance.

Measurements with microelectrodes in tight absorptive epithelia have generally shown a cellular Cl activity greater than that predicted for passive distribution (e.g., frog skin, Nagel, Garcia-Diaz & Armstrong, 1981; rabbit colon, Wills, 1985). Direct microelectrode measurements of cellular Cl activity are not available for the toad urinary bladder. However, estimates of Cl concentration from x-ray microanalysis (Rick et al., 1978; Bowler, Purves & Macknight, 1991), together with recent measurements of membrane potentials (Nagel & van Driessche, 1989; Donaldson & Leader, 1992), indicate that Cl must also be actively accumulated by these epithelial cells. For example, Bowler et al. (1991) estimated a cell Cl concentration of 57 mM in tissues incubated in NaCl Ringer. Assuming that the activity coefficient for Cl in the cells approximates that of the external solution (0.76), an intracellular Cl activity of ~43 mM is obtained. This compares to an expected activity range of 9 to 4 mM if Cl was distributed passively across a membrane with a potential difference of –60 to –80 mV.

The mechanisms involved in Cl accumulation in tight epithelia remain to be established. A range of basolateral membrane Cl channels has been identified in tight epithelia (e.g., rabbit urinary bladder, Hanrahan, Alles & Lewis, 1985; turtle colon, Chang & Dawson, 1988). For Cl to be accumulated in the cell above equilibrium, influx of Cl by secondary active transport must exceed efflux through such basolateral Cl channels. In

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general, three pharmacologically distinct mechanisms have been implicated in the accumulation of Cl in both epithelial and nonepithelial cells; a loop diuretic-sensitive NaK2Cl cotransporter (Geck & Heinz, 1986; O'Grady, Palfrey & Field, 1987), a stilbene disulfonate-sensitive Cl/HCO_3 exchanger (Jennings, 1992), and a thiazide-sensitive NaCl cotransporter (Stokes, 1984; Shimizu et al., 1988). In frog skin, NaK2Cl cotransporters (Dörge et al., 1985; Ussing, 1985) and/or Cl/HCO_3 exchangers (Stoddard, Jakobsson & Helman, 1985), localized to the basolateral membrane, are thought to be responsible for the accumulation of cellular Cl.

In toad urinary bladder, the apical membrane of the epithelial cells has a very low Cl permeability (Macknight, 1977). Therefore, mechanisms responsible for the accumulation of Cl in this tissue are likely to be located in the basolateral membrane. But attempts to identify possible transporters have been singularly unsuccessful in that none of the known inhibitors of the putative transporters has any profound effects on short-circuit current under steady-state conditions in tissues bathed with Na-Ringer. For example, DIDS (4,4′-diisothiocyano-stilbene-2,2′-disulfonate) or SITS, which inhibit Cl/HCO_3 exchangers, and furosemide, which blocks cation-Cl cotransporters, have only a small, pH-sensitive inhibitory effect on baseline I_{sc} and do not inhibit the increase in current induced by vasopressin (Brem et al., 1985). There are no reports of the effects of Cl transport blockers on epithelial cell composition in this tissue.

In the present study we have combined conventional electrical measurements of transepithelial Na transport (e.g., I_{sc}, V_t and R_t) with x-ray microanalysis of cellular composition to examine in detail the relationship between cellular Cl and transepithelial Na transport and to investigate the mechanisms involved in the accumulation of Cl by toad bladder epithelial cells.

The results indicate that a Cl/HCO_3 exchanger is present in the basolateral membrane of the toad bladder and provides an important pathway for Cl movement. In contrast, any NaK2Cl cotransporter appears to be highly variable from tissue to tissue. The results also confirm that the initial inhibition of I_{sc} by serosal Cl-free medium is associated with loss of cell Cl and volume, but thereafter factors other than cell volume modify the relationship between cell Cl and Na transport in this tissue. Changes in pH, associated with the activity of the Cl/HCO_3 exchanger, may be important in this regard.

Materials and Methods

Toads of the species *Bufo marinus,* obtained from the Dominican Republic (National Reagents, Bridgeport, CT) were used in all experiments. For Ussing chamber studies, the serosal surface of quarter bladders was glued with Loctite 454 gel (Loctite, Ireland) to a small plastic ring that was then fitted between the two halves of the Ussing chambers. The serosa was supported on a nylon mesh and a slight excess of mucosal medium held the tissue flat against the mesh. The final exposed area of the quarter bladders was 3.14 cm^2.

Initially, quarter bladders were bathed on both the mucosal and serosal surfaces with NaCl Ringer until a steady-state I_{sc} was obtained. The serosal solution was perfused at a constant rate (3 ml min^{-1}) throughout the experiments, and both the mucosal and serosal solutions were oxygenated and stirred by air bubbled through the chambers.

Tissue for x-ray microanalysis was glued to similar plastic rings and either incubated in small beakers containing Ringer or in Ussing chambers before being frozen. The beakers were bubbled vigorously with air to ensure mixing and oxygenation of the solution. All experiments were carried out at room temperature (15–20°C).

Electrical Measurements

Tissues were short-circuited at all times by a computer-controlled (Apple IIe) automatic voltage clamp. The computer recorded the I_{sc} at 10 or 20 sec intervals. Transepithelial resistance was determined by imposing a potential difference (typically 5 or 10 mV) for 1 sec across the tissue every 2 min, and dividing the applied potential difference by the recorded change in current sampled at the end of the pulse.

Freezing and Cryosectioning

The same techniques were used as described recently (Bowler et al., 1991). Briefly, the plastic ring with its tissue was plunged into a propane-isopentane freezing mixture either by hand or by a spring-loaded device within 20 sec of removal from the incubation medium. Sections were cut at –80 to –90°C. Two or more blocks from each piece of frozen tissue were cut and several sections from each block analyzed. Ribbons of sections on Formvar-coated 3 mm nickel slot grids were transferred into a scanning electron microscope (JEOL JSM-840) with a shuttle device precooled with liquid nitrogen. The microscope stage was precooled below −180°C. Sections were freeze-dried by warming the stage to 16–18°C under vacuum.

Analysis of Sections

Sections were imaged with a transmitted electron detector. Spectra were collected for 100 sec at 15 kV. The probe current (150–250 pA) was measured with a Faraday cup after each spectrum.

Spectra were collected from at least two relatively large areas of albumin above the cells. Data from these areas were pooled to provide an average value. To obtain average cellular values, a large area within each cell, including cytoplasm and a portion of the nucleus, was scanned. Only one measurement was taken from each cell in any section. The data presented come predominantly from analysis of surface cells. In the Dominican toad the vast majority of these are granular cells. Goblet cells are easily identified by the high sulfur peak in the x-ray spectrum and results from these were not included in the data. Mitochondria-rich cells are not readily identified in the unstained, unfixed frozen sections. Although there are relatively few of this cell type it is likely that most of the low Cl cells in Cl medium (e.g., Fig. 3) belong to this category. We did not make a systematic study of the basal cells in this series of experiments.

Methods for data analysis and quantification are described in detail by Bowler et al. (1991). Ion contents are expressed in mmol per kg dry weight (mmol/kg dw).

Solutions

Solutions were prepared fresh as required from analytical grade reagents purchased from Sigma Chemical (St Louis, MO). Sodium chloride Ringer contained (in mM): Na, 116; K, 3.5; Ca, 1.0; Mg, 1.0; Cl, 122.5; SO_4, 1.0; glucose 10, buffered at 7.4 by HPO_4, 2 and H_2PO_4, 0.2. Chloride-free Ringer solutions were prepared from the gluconate salts of Na and K and contained (in mM): Na, 116; K, 3.5; Ca, 1.0; Mg, 1.0; gluconate, 122.5; SO_4, 1.0; glucose 10, buffered at 7.4 by HPO_4, 2 and H_2PO_4, 0.2. Sodium-free Ringer was obtained by equimolar replacement of Na with *n*-methyl-D-glucamine (NMDG). Although gluconate reduces the medium Ca activity (Christofferson & Skibsted, 1975), we did not increase Ca concentration in the media, as in previous studies when Ca was raised to 4.5 mM, no differences were found (Navarte & Finn, 1980, 1983; Lewis et al., 1985).

Stock solutions of bumetanide (a generous gift from Leo Pharmaceuticals) and the thiazides bendroflumethiazide and hydrochlorothiazide (Sigma) were prepared in NaOH, and microliter quantities added to the serosal solution. Stock solutions of SITS, DIDS (Sigma) and amiloride (Merck, Sharp & Dohme, New Zealand) were prepared in distilled water, stored in the dark, and added to the appropriate bath in microliter quantities. Stock solutions of 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (Research Biochemicals) were prepared in DMSO, and added to the appropriate bath in microliter quantities. Arginine vasopressin was obtained from Sigma.

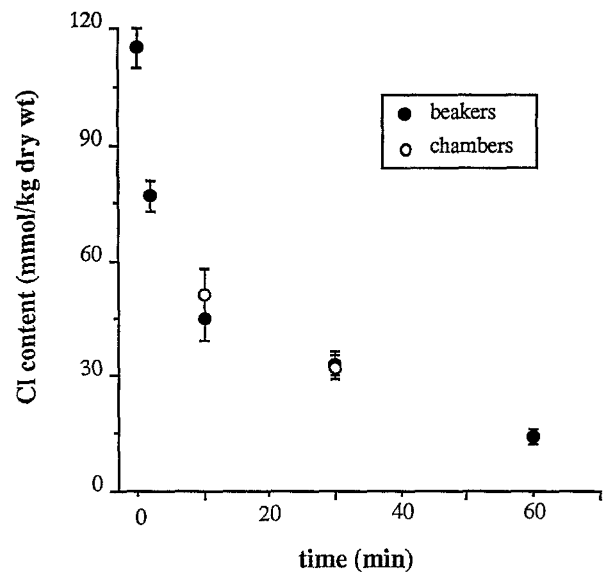

Fig. 1. Time course of loss of Cl into Cl-free gluconate Ringer. Filled circles from tissues incubated in beakers. Open circles are from tissues mounted in Ussing chambers and exposed to Cl-free Ringer on serosal side only. Each sample represents the mean of at least 19 cells.

Results

Effect of Cl-free Ringer on Cell Cl and Na Transport

Replacement of Mucosal or Serosal Cl

Initial experiments compared the effects of Cl-free mucosal medium, Cl-free serosal medium and Cl-free mucosal and serosal medium on the cellular Cl content. Tissues were mounted in Ussing chambers and incubated in the appropriate Ringer for 30–40 min before being frozen for analysis.

The loss of Cl across the apical membrane was minimal (Cl-free mucosa, Cl = 122 ± 5 mmol/kg dw [n = 66] compared to 146 ± 5 mmol/kg dw [n = 113] in NaCl Ringer). In addition, incubation in Cl-free mucosal and serosal Ringer for 30 to 40 min (Cl = 45 ± 3 mmol/kg dw; n = 116) resulted in no greater loss of Cl than incubation in Cl-free serosa alone (Cl = 43 ± 3 mmol/kg dw; n = 135).

Time Course of Cl Loss

The time course of the Cl loss in gluconate medium was determined by placing quarter bladders attached to small plastic rings into beakers for set intervals. In this set of experiments cellular Cl fell to 14 ± 2 mmol/kg dw (n = 32) after 60 min (Fig. 1, filled circles). No further loss of cellular Cl was evident in tissues incubated for 120 min (15 ± 3 mmol/kg dw; n = 34). That the predominant loss of Cl occurred across the basolateral membrane was confirmed by measuring the rate of Cl loss in tissues mounted in Ussing chambers and bathed with Cl-free Ringer on the serosal side only for 10 and 30 min. Cell Cl losses at these times under these conditions were virtually identical to those measured in the beakers (Fig. 1, open circles).

Na Transport

Since Cl loss across the apical membrane was minimal in Cl-free medium, to investigate the effects of cellular Cl loss and recovery on Na transport, only the serosal solution was replaced with gluconate Ringer. This avoided the unrelated inhibitory effects of mucosal gluconate on Na transport (Lewis et al., 1985).

Replacement of serosal Cl with gluconate eventually inhibited I_{sc} in all experiments (e.g., Fig 4*A*). In approximately half of the experiments, inhibition was preceded by an initial small transient stimulation (e.g., Fig. 4*A*). Apart from this variable, initial transient stimulatory effect of gluconate, all tissues behaved identically. Therefore, results from all tissues are included in the following analysis. On average, in serosal gluconate, the I_{sc} fell to 21 ± 1% of the initial I_{sc} within 11 min (Table 1). Associated with the fall in I_{sc} was a slight increase in R_t from 7.5 ± 0.4 to 8.7 ± 0.4 kΩ · cm^2. The I_{sc} then recovered progressively throughout exposure to serosal gluconate, reaching 44 ± 5% of the initial I_{sc} after 30 min. Over this period there were no appreciable changes in transepithelial resistance. (Tissues exposed to both

Table 1. Summary of the effects of replacement of serosal Cl with gluconate on the I_{sc} of the toad bladder

	I_{sc} ($\mu A \cdot cm^{-2}$)	R_t ($k\Omega \cdot cm^2$)
Initial	5.4 ± 0.4	7.5 ± 0.4
Serosal NaCl-Na gluconate		
Minimum	1.2 ± 0.3	8.7 ± 0.4
After 35 min	2.5 ± 0.3	8.1 ± 0.4
Serosal Na gluconate—NaCl		
Peak	9.5 ± 1.1	5.8 ± 0.5
Steady-state	7.4 ± 0.7	5.5 ± 0.4

All values mean ± SEM, $n = 42$.

serosal and mucosal gluconate displayed the same pattern; *data not shown.*)

Reintroduction of serosal Cl resulted in an initial rapid, relatively small rise in I_{sc} to a plateau value within 2 min. An appreciable part of this increase can be accounted for by differences in the mobility of Cl and gluconate through the paracellular pathway, as tissues treated with amiloride also showed an increase in I_{sc} under these conditions (Fig. 4*A*). Thereafter, there was a further increase in I_{sc} to a peak value after 9 min followed by a gradual decline until a new steady-state value, 44 ± 11% higher than the initial I_{sc} was achieved. Accompanying these changes in I_{sc} was a reduction in R_t and, in the steady-state after reintroduction of Cl, R_t was 75 ± 4% of its initial value.

Changes in Ion Content Following Replacement of Serosal Cl with Gluconate

The average water and electrolyte contents of cells incubated in either NaCl Ringer for 90 min, Cl-free serosal Ringer for 30–40 min, or Cl-free Ringer for 30 min followed by NaCl Ringer for 60 min are illustrated in Fig. 2*A*. In this series of experiments, the replacement of medium Cl resulted in a fall of Cl from 147 ± 6 mmol/kg dw ($n = 72$) to 26 ± 3 mmol/kg dw ($n = 71$) after 30–40 min. Cells also lost cation, principally K. However, this cation loss (61 mmol/kg dw) was only about half of the Cl loss (Fig. 2*B*). Water loss accompanied these losses of solute (controls 3.58 ± 0.15 kg/kg dw, Cl-free 2.93 ± 0.15 kg/kg dw) and analysis of the solute and water losses indicates that, within the error of the estimates, the fluid loss was essentially isosmotic (280 mOsmol l^{-1} compared to estimated medium osmolarity of 262 mOsmol l^{-1}). Restoration of medium Cl after 30–40 min essentially restored cell composition.

The patterns of Cl distribution (Fig. 3) did not differ appreciably in control tissues incubated in NaCl medium and in tissues incubated in Cl-free medium and then returned to NaCl medium. In both groups of tissues there were a number of cells with a very low Cl content. These are most likely mitochondria-rich cells (Rick, Spancken & Dörge, 1988; Bowler et al., 1991).

Mechanisms of Basolateral Cl Movement

The Cl content of the granular epithelial cells represents a balance between active accumulation and passive leak. The low Cl permeability of the apical membrane relative to the basolateral membrane of the granular cells localizes both the accumulative and leak pathways to the basolateral membrane. In general, three mechanisms have been implicated in the accumulation of Cl by both epithelial and nonepithelial cells; thiazide-sensitive NaCl cotransport, loop diuretic-sensitive NaCl (or more likely NaK2Cl) cotransport and SITS- and DIDS-sensitive Cl/HCO_3 exchange. Both cotransporters are directly linked to Na and result in the movement of Na and Cl across the basolateral membrane. The Cl/HCO_3 exchanger is functionally linked to Na/H exchange via cellular pH and the collective activity of these two transporters also results in Na and Cl transport. In contrast, Cl channels contribute to Cl loss.

Effects of Na-free Medium

Initially we attempted to exploit the Na-dependency of these three Cl transporters to demonstrate the involvement of a Na-dependent process in the accumulation of Cl. Removal of serosal Na is known to inhibit I_{sc} (Macknight, DiBona & Leaf, 1980) and did so in these experiments. However, this inhibition was not due to a loss of cellular Cl or to a decrease in cellular volume. In fact, when serosal Na was replaced with NMDG, cell Cl increased slightly from 122 ± 3 ($n = 186$) to 143 ± 5 mmol/kg dw ($n = 70$), whereas cell water content remained essentially unchanged. Under these conditions cell Na decreased from 61 ± 3 to 42 ± 4 mmol/kg dw. This was compensated for by an increase in cell K (from 465 ± 6 to 493 ± 8 mmol/kg dw).

In frog skin, despite the presence of a NaK2Cl cotransporter (Dörge et al., 1985; Ussing, 1985), Na-free serosal medium also has little effect on cellular volume (Ussing, 1982) or cellular Cl (Dörge et al., 1985). In both tissues this may be due to very low levels of activity of the pathways contributing to the Cl permeability of the basolateral membrane under normal conditions, as suggested for frog skin (Ussing, 1982). Therefore, in subsequent experiments we investigated the effects of Cl transport inhibitors on the changes in I_{sc} and cellular electrolyte content that occurred with either incubation in Cl-free medium or following reintroduction of serosal Cl after depletion of cellular Cl through exposure to serosal Cl-free Ringer.

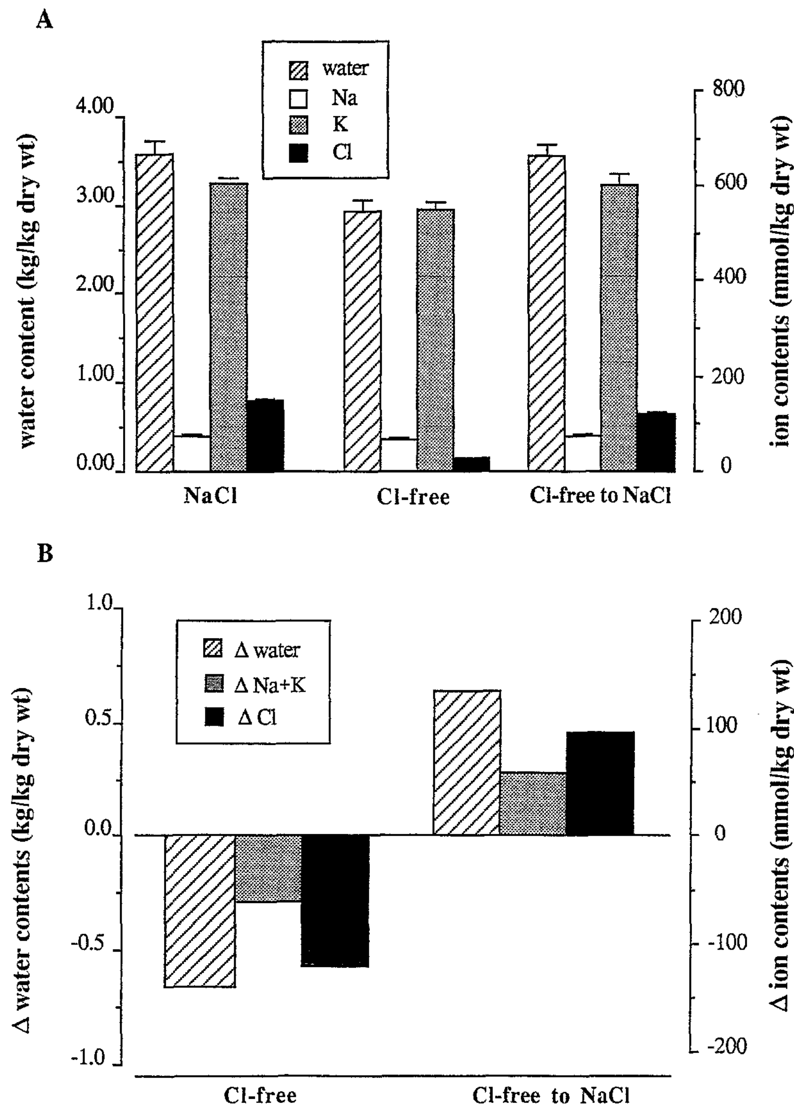

Fig. 2. Water and ion contents determined by x-ray microanalysis. (*A*) Average water and ion contents of toad bladder epithelial cells incubated in NaCl Ringer, in Cl-free gluconate Ringer or Ringer in which Cl was restored. Error bars indicate SEM. (*B*) Changes in water and ion content of epithelial cells when Cl-free gluconate Ringer replaced NaCl Ringer and when Cl was restored to the medium.

Effects of Cl Transport Blockers on Transepithelial Na Transport

Whether added alone or in combination there was no effect of serosal SITS (0.5 mM), bumetanide (0.1 mM) or thiazides (hydrochlorothiazide and bendroflumethiazide, 0.1 mM) on the inhibition of I_{sc} by serosal gluconate. Also, neither of the thiazides (0.1 mM) had an effect on recovery of I_{sc} in Cl Ringer.

The addition of SITS (Fig. 4*B*) to the serosal bath inhibited the gradual secondary recovery of I_{sc} in gluconate Ringer. In the control group for this series, the I_{sc} increased over a 20 min period from a minimum value of 1.7 ± 0.5 $\mu A \cdot cm^{-2}$ to 2.9 ± 0.7 $\mu A \cdot cm^{-2}$ (n = 12). In the experimental tissues exposed to SITS the I_{sc} fell to a minimum (1.29 ± 0.29 $\mu A \cdot cm^{-2}$), then recovered briefly before gradually declining so that, after 20 min in gluconate medium, the mean I_{sc} was 1.16 ± 0.27 $\mu A \cdot cm^{-2}$ (n = 12).

SITS also inhibited the recovery of I_{sc} when Cl Ringer replaced serosal gluconate Ringer. The degree of this inhibition, however, was quite variable. In 2 of the 12 tissues, SITS had no effect on the recovery of I_{sc}. In another two tissues, there was only a small increase in I_{sc} with the reintroduction of Cl, comparable to that seen in tissues in which transport was inhibited by mucosal amiloride (Fig. 4*A*). This suggests that much of the increase was due to the differences in mobility of Cl and gluconate through the paracellular pathway. A representative example of how the remaining eight tissues were affected by SITS under these conditions is shown in Fig. 4*B*. In these tissues there was a partial inhibition of the recovery of I_{sc}. Generally, there was a much more dramatic inhibition of the initial peak increase in I_{sc}, although there was also significant inhibition of the steady-state I_{sc} (e.g., Fig. 4*B*). Lower concentrations of SITS (e.g., 0.1 mM) had no effect on the I_{sc}. It was not possible to reverse the effects of SITS by washing.

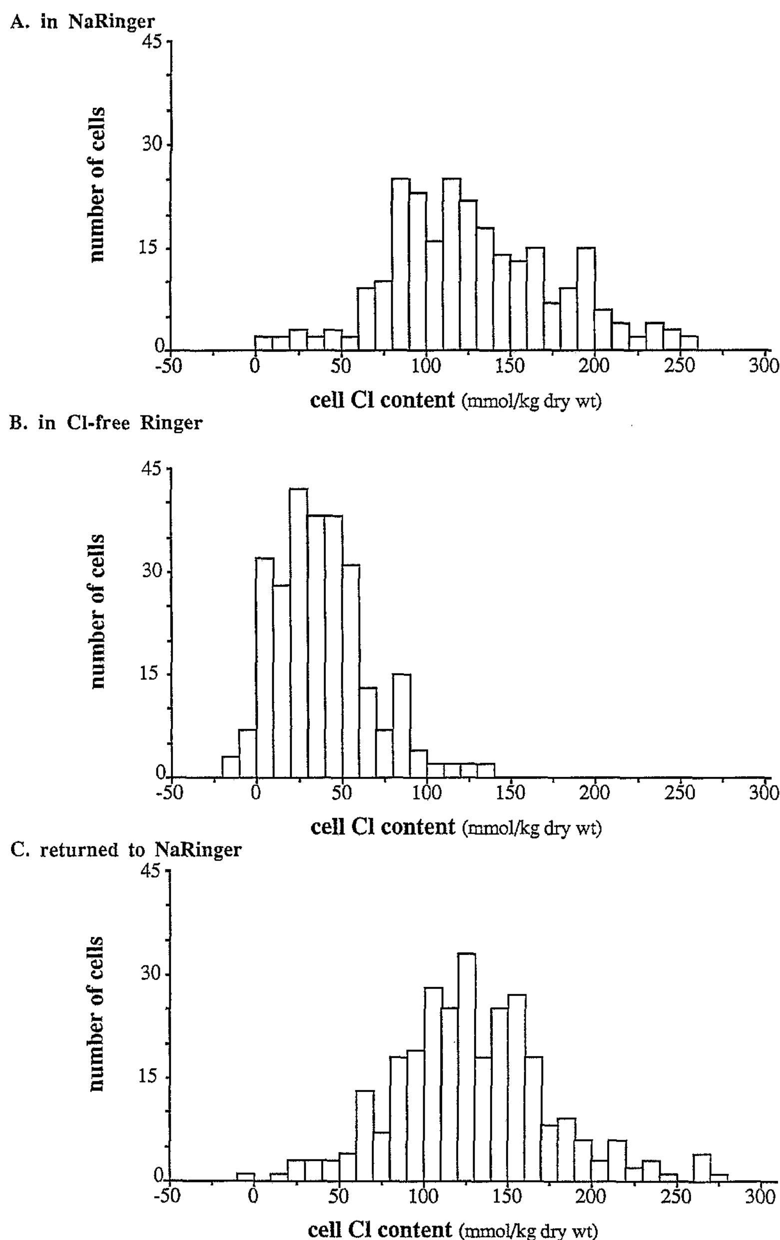

Fig. 3. Frequency distribution of Cl content in toad bladder epithelial cells. (*A*) Bladders incubated in NaCl Ringer throughout. (*B*) Bladders incubated in Cl-free gluconate Ringer. (*C*) Cells incubated in Cl-free gluconate Ringer and then Cl restored to the medium for 60 min.

Bumetanide was less effective than SITS in inhibiting the recovery in I_{sc}. In 11 tissues, serosal bumetanide (0.1 mM) had no discernible effect on either the recovery of I_{sc} in gluconate Ringer or in Cl Ringer, but in 3 tissues, bumetanide inhibited both the recovery of I_{sc} in gluconate Ringer and the subsequent recovery in Cl Ringer (Fig. 4*C*).

Despite the variable effects of SITS and bumetanide when present alone, the combination of serosal bumetanide (0.1 mM) and SITS (0.5 mM) always completely inhibited the recovery of I_{sc} in gluconate and Cl-Ringer (n = 10), apart from the initial slight increase in I_{sc} in Cl-Ringer that results from junctional effects (Fig. 4*D*). The combined actions of these two blockers were not a result of toxic effects on the cells, for measurement of intracellular ions by x-ray microanalysis showed that the Na and K contents of the epithelial cells under these conditions were normal (*see below*). Also, vasopressin resulted in a marked increase in I_{sc} in SITS and bumetanide-treated tissues after the restoration of medium Cl (Fig. 5).

Effect of Cl Transport Blockers on Changes in Cellular Ion Content

The combination of serosal SITS plus bumetanide had the most profound effect on the changes in I_{sc} associated

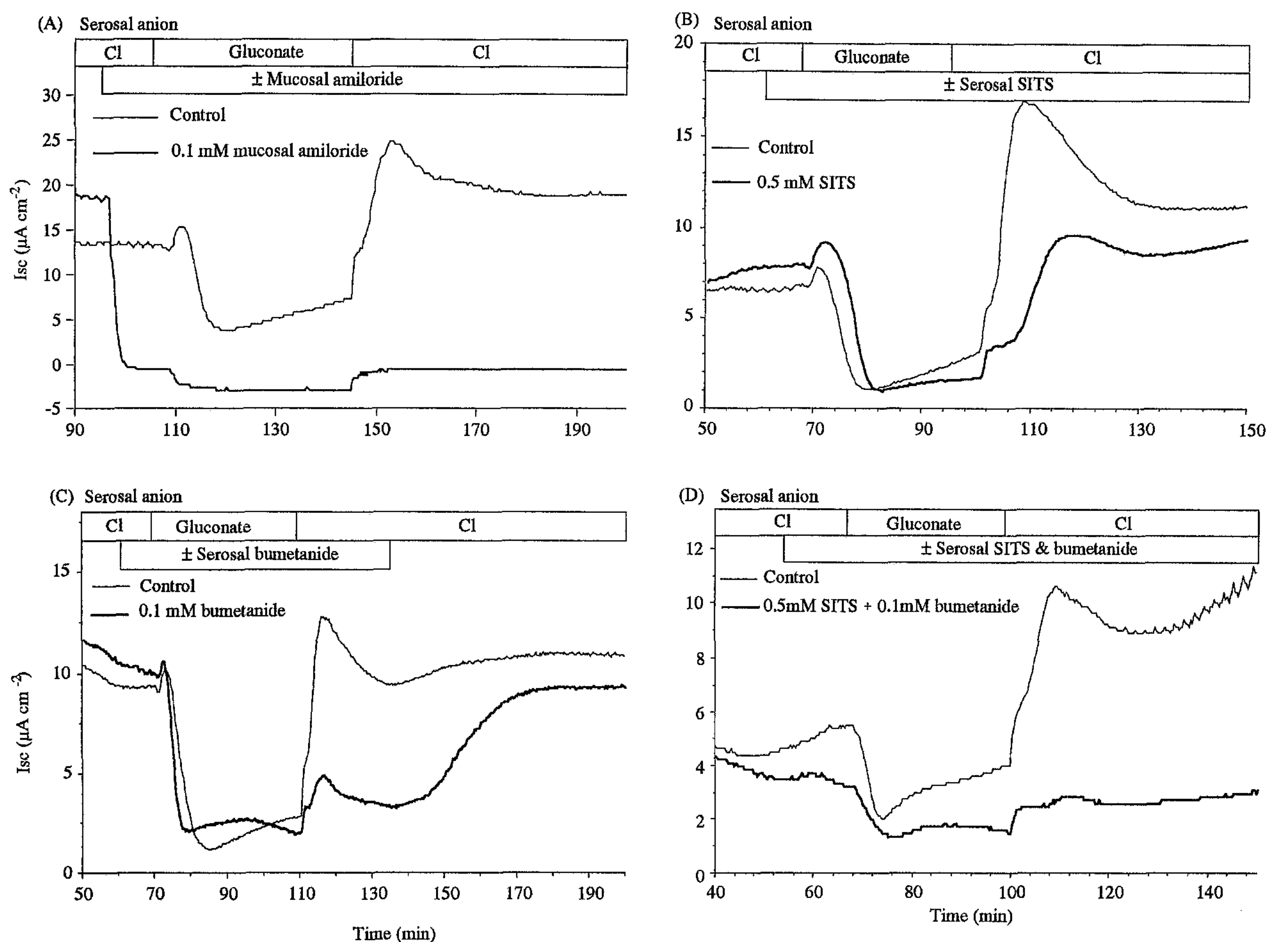

Fig. 4. Examples of the inhibitory effects of amiloride, SITS, and bumetanide on the recovery of I_{sc} in Cl Ringer after exposure to serosal gluconate Ringer for 30 min. (*A*) 0.1 mM mucosal amiloride. All tissues responded in this fashion. (*B*) 0.5 mM serosal SITS. Eight of 12 tissues responded in this fashion. Two of the remaining four tissues were unaffected and two showed a more complete inhibition as seen with the combination of SITS and bumetanide. (*C*) 0.1 mM serosal bumetanide. Only 3 of 14 tissues responded in this fashion. In the remaining 11 tissues, no inhibition was seen. (*D*) 0.5 mM SITS plus 0.1 mM bumetanide added to the serosal solution simultaneously. All 10 tissues responded in this fashion.

with serosal Cl-free Ringer. Initially, we therefore investigated whether this combination of blockers affected the changes in cellular ion contents which accompanied the variations in I_{sc} (Table 2).

As shown above (Figs. 1 and 2), in control tissues the replacement of serosal Cl with gluconate for 30 min resulted in a significant fall in cellular Cl and, following the reintroduction of Cl, the cells recovered the lost Cl within 10 min. Serosal bumetanide and SITS failed to prevent the loss of Cl. Furthermore, when Cl was reintroduced the tissues exposed to the blockers reaccumulated Cl at the same rate and to the same extent as the controls. Thus, these blockers, despite markedly inhibiting the I_{sc}, did not inhibit the recovery of cellular Cl. Neither did 0.1 mM bumetanide, 0.5 mM SITS or 0.1 mM thiazide individually (*data not shown*).

Despite the low Cl permeability of the apical membrane under normal conditions (*see above* and Macknight, 1977), it was possible that the modified conditions used here altered the apical Cl permeability so that, in the presence of serosal blockers, there was significant Cl accumulation across the apical membrane. To test this possibility, two experimental protocols were carried out. In the first, cells were depleted of Cl by incubating them in mucosal and serosal Cl-free medium and then Cl was reintroduced to the serosal medium only. Tissues treated in this fashion had similar Cl levels (154 ± 6 mmol/kg dw) as tissues bathed in mucosal and serosal Cl Ringer throughout (147 ± 6 mmol/kg dw).

In the second protocol, the cells were depleted of Cl in mucosal and serosal Cl-free Ringer, bumetanide and SITS were then added to the serosal solution in an attempt to minimize loss of Cl across that membrane, and then the mucosal solution was replaced with Cl Ringer. In these tissues the cellular Cl levels (55 ± 5 mmol/kg dw) were significantly lower than in the controls and comparable to those found in tissues in this set of experiments after depletion of cellular Cl in Cl-free Ringer (45

Table 2. Effects of a combination of 0.5 mM serosal SITS and 0.1 mM serosal bumetanide on the changes in cellular water and ion contents associated with exposure to serosal Na gluconate Ringer for 30 min and then returning to serosal NaCl Ringer for periods of 10 or 60 min

	(n)	H_2O kg/kg dw	Na	K	Cl	P
			mmol/kg dw			
NaCl Ringer throughout	(72)	3.58 ± 0.10	73 ± 5	605 ± 10	147 ± 6	682 ± 17
Serosal Cl-free Ringer						
30 min	(71)	2.93 ± 0.12	65 ± 5	552 ± 13	26 ± 3	667 ± 15
30 min plus blockers	(74)	2.84 ± 0.08	58 ± 3	518 ± 10	28 ± 3	665 ± 17
Serosal Cl Ringer						
10 min	(43)	3.42 ± 0.15	69 ± 6	582 ± 13	86 ± 4	733 ± 22
10 min plus blockers	(95)	3.45 ± 0.11	51 ± 4	603 ± 15	95 ± 4	699 ± 15
60 min	(55)	3.56 ± 0.15	74 ± 7	601 ± 19	120 ± 6	683 ± 17
60 min plus blockers	(60)	3.31 ± 0.12	65 ± 6	605 ± 17	128 ± 7	690 ± 20

Mucosal solution throughout NaCl Ringer. All values Mean ± SEM, n = number of cells analyzed from at least three bladders.

± 3 mmol/kg dw). Thus, there was no evidence for a significant apical membrane Cl pathway under these experimental conditions.

Effect of the Cl Channel Blocker, NPPB

It is likely that the continued loss of Cl in the presence of bumetanide and SITS is a consequence of loss via Cl channels. In recent years a range of Cl channel blockers has been developed (Wangemann et al., 1986; Greger, 1991), of which the most effective blocker of epithelial Cl channels appears to be the compound NPPB (Wangemann et al., 1986). In an attempt to examine the role of Cl channels in the movement of Cl across the basolateral membrane of the toad bladder, we assessed the effects of serosal NPPB (50 μM), with or without bumetanide and SITS, on the changes in I_{sc} and cellular ion content that occurred with either incubation in Cl-free medium or following the reintroduction of serosal Cl after depletion of cellular Cl through exposure to serosal Cl-free Ringer.

Whereas the combination of SITS and bumetanide had minimal effect upon the I_{sc}, the inclusion of NPPB markedly inhibited the I_{sc} in NaCl Ringer (a 60% reduction in I_{sc} over 10 min). This effect appeared to result from metabolic inhibition as, in tissues incubated with NPPB, either alone or in combination with SITS and bumetanide, up to one third of the cells had ion patterns consistent with metabolic inhibition (i.e., high Na, low K and elevated Cl).

Discussion

Chloride is present in the cells of model absorptive tight epithelia, such as the toad urinary bladder and frog skin, at concentrations above equilibrium distribution (Rick et al., 1978; Nagel et al., 1981). In the toad bladder it is unclear what the mechanism(s) responsible for the accumulation of Cl are. Our primary objective in this study was to use x-ray microanalysis of granular cell composition and recognized pharmacological inhibitors of Cl transporters to determine how Cl is accumulated by the granular cells of the toad bladder. For the reasons discussed below we conclude that the dominant mechanism for this accumulation is a SITS-sensitive Cl/HCO_3 exchanger. Also, the results indicate that changes in cellular Cl content and I_{sc} can be dissociated, suggesting that in the toad bladder there is no simple relationship between cell Cl, cellular volume and I_{sc}.

Relationship between Cellular Cl and Na Transport

In both toad bladder (Lewis et al., 1985) and frog skin (Leibowich et al., 1988), the inhibition of I_{sc} following replacement of serosal Cl with an impermeant anion, such as gluconate, is thought to be a consequence of changes in cell volume, with the loss of cellular Cl and accompanying cation and water resulting in cellular shrinkage. It has been proposed that the shrinkage in turn inhibits basolateral K channels, depolarizing the cell membrane, thereby reducing the driving force for Na entry (Lewis et al., 1985).

The results of the present study are consistent with a role for cellular shrinkage in the inhibition of I_{sc}. Following replacement of serosal Cl with gluconate, cellular Cl fell from 147 ± 2 to 28 ± 3 mmol/kg dw over a period of 30 min. Associated with this was a reduction in cell water from 3.58 ± 0.1 to 2.93 ± 0.12 kg/kg dw. The inhibition of I_{sc} paralleled the loss of cell Cl and water. When the I_{sc} reached a minimum 11 min after the replacement of Cl with gluconate, cellular Cl had fallen to 45% of its original value.

Although the initial inhibition of I_{sc} in Cl-free medium appeared to be closely related to the loss of cell Cl and cellular shrinkage, subsequent changes in I_{sc}, both

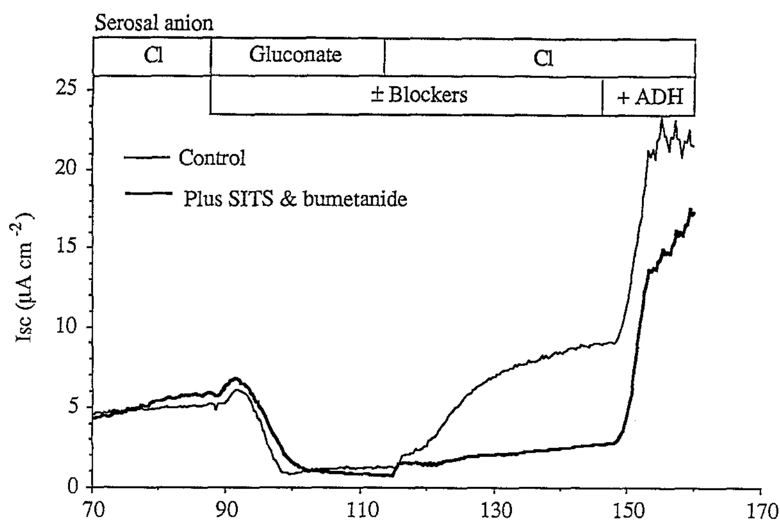

Fig. 5. Changes in I_{sc} in response to serosal vasopressin (1 mIU ml^{-1}) following inhibition of the recovery of I_{sc} in Cl Ringer by serosal SITS (0.5 mM) plus bumetanide (0.1 mM) after exposure to serosal gluconate Ringer.

while the tissue remained in gluconate medium and when Cl was reintroduced, do not appear to be closely coupled to cell Cl and volume. Indeed at times there was no obvious relationship. For example, after the I_{sc} reached a minimum value in gluconate medium, significant recovery of the I_{sc} occurred despite continued incubation of the tissue in gluconate medium and continued loss of cellular Cl. Thus, although I_{sc} fell to 21% of the initial I_{sc} 11 min after the replacement of Cl, 20 min later the I_{sc} had recovered to 45% of the original I_{sc}. During this period the cells lost a further 20% of their cellular Cl (50 to 30 mmol/kg dw). Also, following recovery in Cl medium the steady-state I_{sc} was significantly higher than the initial I_{sc} despite similar levels of cellular Cl. In contrast, if the inhibitors SITS and bumetanide were present, Cl recovered to levels similar to controls but the I_{sc} did not recover at all.

These observations suggest that factors other than changes in cellular volume modify the initial inhibition of I_{sc} by cellular shrinkage and contribute significantly to the recovery of I_{sc} on the reintroduction of medium Cl. It is not possible to determine directly from the measurements obtained in this study which factors are involved. However, given that a Cl/HCO_3 exchanger provides an important pathway for the movement of Cl across the basolateral membrane (*as discussed below*) variations in cytoplasmic pH may play a role.

In frog skin bathed in Ringer containing HCO_3, loss of cell Cl in exchange for HCO_3 ions alkalinizes the cell (Civan, Cragoe & Peterson-Yantorno, 1988) and alkalinization activates both the basolateral membrane K conductance and apical membrane Na conductance (Harvey, Thomas & Ehrenfeld, 1988). In toad bladder a similar alkalinization due to Cl loss in exchange for HCO_3 would counteract the shrinkage-induced inhibition of I_{sc}. This is consistent with the observed recovery of I_{sc} in gluconate medium (Table 1, Figs. 4 and 5). Similarly, the response of I_{sc} to restoration of Cl is consistent with an increase in basolateral K conductance in response to cell volume at a time when alkalinization has already increased the basolateral K and apical Na conductances. Thus, at about 10 min after reintroduction of Cl, I_{sc} peaked at a value 175% of initial. Thereafter it declined to a steady-state value of 136% of initial, consistent with a reduction in the alkalinization effect through the activity of the Cl/HCO_3 exchanger.

With SITS plus bumetanide any activity of the Cl/HCO_3 and NaK2Cl cotransporters will be inhibited. Inhibition of the Cl/HCO_3 exchanger during the loss of Cl into gluconate medium would reduce the alkalinization accompanying removal of Cl. Under these conditions, shrinkage-induced inhibition of the basolateral K conductance would not be countered to the same extent by increased cytoplasmic pH. Also, the apical membrane Na conductance would not be activated. This would explain the failure of I_{sc} to drift upwards following the initial depression in Cl-free medium. When Cl is returned to the serosal medium, the apical Na and basolateral K conductances are inhibited and I_{sc} does not recover, despite the increase in cellular Cl and volume. Indeed, the continued inhibition of the basolateral K conductance and associated cellular depolarization under these conditions provides an explanation for the increase in cellular Cl (*see below*).

The above arguments are supported by evidence from other studies in toad bladder which show that serosal pH affects I_{sc}, acidification of this medium inhibiting transport and alkalinization stimulating transport (Carvounis, Levine & Hays, 1979), and that SITS, DIDS and furosemide inhibited I_{sc} in NaCl Ringer at a serosal pH of 7.1 but not at 8.5 (Brem et al., 1985).

Although the data reported in the present study suggest that variations in cytoplasmic pH modify the response of the bladder to changes in cell volume, changes in other cytoplasmic factors may be involved. For example, variations in Ca activity modify the response of

Na channels from the toad bladder to variations in pH (Garty, Asher & Yeger, 1987).

Mechanisms Involved in the Loss and Accumulation of Cl

Pathways involved in either the accumulation or loss of Cl by the granular cells must be localized in the basolateral membrane, as x-ray microanalysis showed little change in cellular Cl with changes in mucosal medium Cl. This confirms earlier ^{36}Cl flux studies (Macknight, 1977).

The activity of the basolateral membrane transport pathways appears to be minimal under control conditions. Specific inhibitors of Na-dependent cotransporters and Cl/HCO_3 exchangers have little effect upon the I_{sc} under normal conditions (Brem et al., 1985; *personal observation*) and replacement of serosal Na with *n*-methyl-D-glucamine, despite inhibiting the I_{sc}, had no effect upon the cellular Cl content or volume. Similar observations have been reported for the frog skin (Ussing, 1982; Dörge et al., 1985), and Ussing (1982) has suggested that in this tissue under steady-state conditions the low levels of activity of the Cl channels and Cl transporters ensure that little work is required to maintain the nonequilibrium distribution of the Cl ion.

The low levels of activity under normal conditions led us to investigate the extent of alterations in cell composition resulting from Cl-free conditions, or alternatively the extent of recovery of cell Cl when serosal Cl was restored. These measurements indicate that Cl/HCO_3 exchange is an important mechanism for Cl movement across the basolateral membrane. In contrast there was no evidence of thiazide-sensitive NaCl cotransport contributing to Cl transport, and any contribution of diuretic-sensitive NaK2Cl cotransport appeared to be highly variable from bladder to bladder.

Evidence for a Cl/HCO_3 Exchanger

It is apparent that the loss of cellular Cl in Cl-free gluconate medium markedly exceeds the loss of cellular cation (Fig. 2*B*). In Cl-free medium, the cells lost 121 mmol/kg dw Cl. The cation loss was only half of this (K loss 53 mmol/kg dw, Na loss 8 mmol/kg dw). These changes in ion content were completely reversed on reintroduction of Cl to the serosal medium. Despite the inequality in the alterations of cation and anion contents under these conditions, the changes in cellular water that occurred were those expected for an essentially isosmotic loss of Na, K and Cl.

This imbalance between the loss (and recovery) of Cl and accompanying cation requires alterations in the net charge on other cellular solutes. Variations in cell HCO_3 arising from loss (and recovery) of Cl via a Cl/HCO_3 exchanger would achieve this. In the presence of an impermeant external anion, such as gluconate, loss of Cl via a Cl/HCO_3 exchanger would drive HCO_3 into the cell where it would react with H ions to form water and CO_2, thus alkalinizing the cell. The net effect would be to decrease the H ions buffered by negative charges on nondiffusible intracellular anions, leaving these negative charges to be balanced predominantly by K. Thus, some of the K previously balancing charge on Cl ions lost from the cells would be retained within the cells. With the reintroduction of Cl, HCO_3 ions would be removed from the cell via the Cl/HCO_3 exchanger. The companion H ions would be buffered on cellular negative charges, freeing cell K to balance charge on some of the accumulated Cl.

The effects of SITS on the recovery of I_{sc} in Cl medium after depletion of cellular Cl in serosal gluconate medium are in accord with the conclusion that a Cl/HCO_3 exchanger is involved in the accumulation of Cl. In 10 of 12 tissues, SITS either completely or partially inhibited the recovery of I_{sc}. Similarly, SITS with bumetanide modified the changes in cellular ion content associated with removal or reintroduction of Cl in a fashion consistent with the inhibition of a Cl/HCO_3 exchanger. Cells exposed to Cl-free medium containing these inhibitors lost similar amounts of Cl (119 mmol/kg dw) and measured cations (102 mmol/kg dw), predominantly K. Again, the water loss was consistent with isosmotic loss of the measured ions and, when Cl was restored, cells gained comparable amounts of Cl (100 mmol/kg dw) and cation (94 mmol/kg dw).

Evidence for a NaK2Cl Cotransporter

There is little evidence for the involvement of a NaK2Cl cotransporter in the movement of Cl across the basolateral membrane. Bumetanide, a recognized inhibitor of NaK2Cl cotransport in a range of tissues (Geck & Heinz, 1986; O'Grady et al., 1987) did not modify granular cell loss or recovery of Cl. However, in 3 of 12 tissues bumetanide alone did inhibit the recovery of I_{sc}, both in gluconate Ringer and with the reintroduction of Cl. Furthermore, it appeared to potentiate the ability of SITS to inhibit the recovery of I_{sc}, particularly with the reintroduction of Cl. In the presence of both bumetanide and SITS the recovery of I_{sc} following the reintroduction of Cl was always completely inhibited.

Whether these effects of bumetanide resulted from inhibition of NaK2Cl cotransport is unclear for they may reflect a nonspecific action on the Cl/HCO_3 exchanger. The concentrations of bumetanide used in this study do inhibit Cl/HCO_3 exchange in other tissues (Jennings, 1992). Furthermore, it is difficult to explain the inhibition by bumetanide of the recovery of I_{sc} in gluconate medium, other than by an action on the SITS-sensitive pathway.

If the effects of bumetanide on recovery of I_{sc} do reflect inhibition of the NaK2Cl cotransporter, particularly following the reintroduction of Cl, then the results suggest that the contribution of NaK2Cl cotransport to total Cl uptake is variable. This would explain the variable response of the tissue to either SITS or bumetanide alone. In those tissues where Cl/HCO_3 activity is dominant then SITS, but not bumetanide, would prevent the recovery of I_{sc}. In contrast, where the contribution of the NaK2Cl cotransporter was more significant, inhibition of the Cl/HCO_3 exchanger with SITS would be less likely to affect the recovery of I_{sc}, while inhibition of the NaK2Cl cotransporter with bumetanide would inhibit recovery.

Why then was the combination of SITS and bumetanide unable to inhibit either the loss or recovery of Cl? Loss of Cl in the presence of the blockers most likely occurs via Cl channels. While there is no recorded measurement of a Cl conductance in the basolateral membrane of the toad bladder, Cl channels or a Cl conductance have been identified in the basolateral membrane of a range of other tight absorptive epithelia (Hanrahan et al., 1985; Chang & Dawson, 1988).

It is less apparent how Cl is reaccumulated in the presence of the blockers. A novel Cl accumulative mechanism may be present. Alternatively, Cl may be accumulated via Cl channels. Cellular shrinkage associated with loss of cell Cl in gluconate medium reduces the basolateral K conductance of the cells (Lewis et al., 1985). Furthermore, there is evidence that in Cl-free gluconate medium cells depolarize (e.g., in frog skin the cells depolarize from −69 mV in Cl medium to −32 mV in gluconate medium; Leibowich et al., 1988). If similar changes in potential occurred in toad bladder cells, and the cells remained depolarized when Cl was restored in the presence of bumetanide and SITS, cell Cl concentration at equilibrium would approximate 35 mM, a value comparable to the estimated concentration under these conditions.

Confirmation of the role of Cl channels is dependent upon the availability of a suitable Cl channel blocker. In the frog skin, when the Cl channel blocker diphenyl-2-carboxylate (DPC) was added with bumetanide, loss of cellular Cl was prevented although bumetanide alone inhibited the recovery of Cl (Dörge et al., 1989). Unfortunately, NPPB, one of the most effective blockers of epithelial Cl channels, inhibited both transport and metabolism in the toad bladder. Nonspecific effects of NPPB have been reported in other epithelia (Reinach & Schoen 1990; Illek et al., 1992) and NPPB is reported to have a metabolic effect due to mitochondrial uncoupling (Lukacs et al., 1991). Also, it is evident that the effectiveness of this compound varies from tissue to tissue (Greger, 1991). Therefore, before the role of Cl channels can be tested, other techniques will be needed to define an appropriate Cl channel blocker.

In summary, in the absence of inhibitors the replacement of serosal medium Cl with the impermeant anion gluconate was associated with a reversible loss of cellular Cl and water. Accompanying the loss of cell Cl and water there was reversible inhibition of I_{sc}. Although the inhibition of I_{sc} was closely associated with shrinkage of the cells, the recovery of I_{sc} could be dissociated from changes in cell Cl and water. In gluconate medium I_{sc} recovered despite continued loss of cell Cl. Furthermore, the inhibitors SITS and bumetanide prevented the recovery of I_{sc} on reintroduction of serosal medium Cl but did not prevent the recovery of cellular Cl and water. The effects of SITS on the changes in I_{sc} and cellular Cl content that occur with replacement of serosal Cl by gluconate, and that follow the reintroduction of serosal Cl, suggest that a Cl/HCO_3 exchanger is the dominant mechanism for the accumulation of Cl across the basolateral membrane of the granular cells in the toad bladder. If a NaK2Cl cotransporter is present, its level of expression or activity varies markedly from bladder to bladder.

This work was supported by a grant from the Medical Research Council of New Zealand. Purchase of equipment was made possible through grants from the Medical Research Council of New Zealand, the Medical Distribution Committee of the Lottery Board, the University Grants Committee, the Telford Trust, the New Zealand Neurological Foundation and the National Heart Foundation. The expert technical assistance of S. Zellhuber-McMillan is gratefully acknowledged.

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