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Short-term Bromide Uptake in Skins of *Rana pipiens*

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Abstract. Intracellular ion concentrations were determined in split skins of *Rana pipiens* using the technique of electron microprobe analysis. Based on the 1 min Br uptake from the apical bath, two types of mitochondria-rich (MR) cells could be distinguished: active cells which rapidly exchanged their anions with the apical bath and inactive cells which did not. Br uptake and frequency of active MR cells were closely correlated with the skin conductance, g_t. Replacing Cl in the apical bath with an impermeant anion significantly lowered g_t and the Br uptake and Na concentration of active cells. Even larger reductions were observed after apical amiloride (0.1 mM). The inhibition of the Br uptake was reversible by voltage clamping (100 mV, inside positive). Cl removal and amiloride also led to some shrinkage of active cells. The results suggest that the active cell is responsible for a large part of g_t. Inactive MR cells had much lower Br and Na concentrations which were not significantly affected by Cl removal, amiloride, or voltage clamping. Principal cells, which represent the main cell type of the epithelium, showed only a minimal Br uptake from the apical side which was not correlated with g_t. Moreover, Cl removal had no effect on the Na, Br, and Cl concentrations of principal cells.

Key words: Intracellular ions — X-ray microanalysis — Sodium — Chloride — Bromide — Frog skin epithelium

Introduction

The frog skin epithelium is a highly complex epithelial tissue with different cell layers and cell types. Its main transport function is the active Na reabsorption from the environment. In vivo, the influx of Na is balanced by the secretion of hydrogen ions, whereas in vitro, with high external Na concentrations, the Na influx is accompanied by the conductive flow of Cl (Larsen, 1991). Active Na transport and passive Cl reabsorption are coupled via the transepithelial potential difference.

Cl may take a transcellular route, via principal and mitochondria-rich (MR) cells, or a paracellular route, through the spaces between the cells. Correlations between the density of MR cells and the Cl current (Voûte & Meier, 1978; Willumsen & Larsen, 1985), cell volume changes during Cl current activation (Larsen, Ussing & Spring, 1987), and the localization of current peaks over MR cells (Foskett & Ussing, 1986; Katz & Scheffey, 1986) support the view that the MR cell is part of the Cl pathway. Principal cells are probably not involved as indicated by the lack of a Cl concentration change after removing Cl from the apical bath (Biber et al., 1985; Rick et al., 1986) and the absence of a change in the apical Cl conductance during activation of the Cl current (Willumsen & Larsen, 1986).

The existence of a transcellular pathway does not necessarily preclude that a large fraction of the Cl current follows a paracellular shunt path as originally proposed by Ussing and Windhager (1964). In fact, the inhibition of the shunt conductance by amiloride is equally well explained by an increase in the tight-junctional resistance (Nagel, 1989) as by an inactivation of the apical Cl channel in MR cells (Larsen et al., 1987). Also, analyses of the intracellular ion concentrations raised some doubts that the MR cell could fully account for the Cl conductance (Nagel & Dörge, 1990).

We have recently shown that different subtypes of

MR cells can be distinguished based on the response of the intracellular ion concentrations to amiloride and ouabain (Rick, 1992). The measurements were performed by electron probe microanalysis on thin freeze-dried cryosections of the isolated frog skin epithelium of *R. pipiens*. In the present study, this technique is used to follow the path of Cl through the epithelium by measuring the short-term Br uptake from the apical bath. According to the results, two types of MR cells can be distinguished: active cells which readily take up Br from the apical bath, and inactive cells which do not. The Br uptake of active cells is closely correlated with g_t, suggesting that this cell type is responsible for the Cl conductance of the skin. Active cells exist in two functional states: a conducting *on* state and a nonconducting *off* state. Transition from the on to the off state is accompanied by significant losses in Na, anions and cell volume.

Materials and Methods

The methods are largely identical to those described before (Rick, 1992). Briefly, frogs of the northern variety of *R. pipiens* were obtained from Lemberger (Oshkosh, WI) and kept at room temperature (22°C) prior to the experiments. The animals were killed by decapitation and doubly pithing. The abdominal skin was carefully dissected and cut into 3–4 pieces. After 90 min incubation with 1 mg/ml type 2 crude collagenase (Worthington Biochemicals, Freehold, NJ), the epithelium was stripped-off from the underlying tissue. The isolated epithelium was glued onto apertures (exposed area 1.6 cm^2) and mounted in Ussing-type incubation chambers for recording of the short-circuit current (SCC) and small signal conductance (g_t).

Initially, the pieces were incubated in frog Ringer solution until the SCC had reached a steady-state. At this time, the apical bath was replaced with Cl-free Ringer or Ringer solution containing 0.1 mM amiloride (a gift from Merck, Sharp & Dohme, Rahway, NJ). In the amiloride experiments, one of the skin pieces was voltage-clamped to 100 mV (inside positive). Another piece remained untreated, serving as a control. After 10-15 min, the Br uptake was initiated by exchanging the apical bath with Br Ringer solution. Shortly before the end of the 1 min uptake period, the epithelium was removed from the chamber and shock-frozen by plunging the aperture sideways in liquid ethane (−188°C).

Normal Ringer solution contained (in mM) 110 NaCl and 1 $CaCl_2$; Cl-free Ringer 55 Na_2SO_4 and 1 Ca gluconate; and Br Ringer 110 NaBr and 1 $CaBr_2$. All media contained 2.5 $KHCO_3$ and 5 glucose and were equilibrated with air (pH of 8.0–8.3).

The tissue was cut dry at −140°C in a cryoultramicrotome (Reichert Ultracut with FC4 D, Vienna, Austria), at a nominal section thickness of 250 nm. The sections were freeze-dried overnight at −90°C and 10^{-7} mbar and transferred to a scanning EM (Stereoscan S250, Cambridge Instruments, Cambridge, UK) which was equipped with a solid-state x-ray detecting system (Link AN10000, High Wycombe, UK) and a custom-made transmission stage. Measuring conditions were 20 kV acceleration voltage, 0.5 nA probe current, and 100 sec analysis time. Analyses were performed in the nucleus and organelle-free areas of the cytoplasm. After film-stripping, the element-characteristic peaks were separated from the noncharacteristic background radiation (Bauer & Rick, 1978).

Quantification of the cellular element concentrations and dry weight fraction was achieved by comparing the x-ray spectra obtained in the cell with those obtained in a peripheral albumin standard layer (Rick, Dûrge & Thurau, 1982). The standard was prepared by dissolving 12 g/dl lyophilized bovine serum albumin (A4378, Sigma, St. Louis, MO) in the Br Ringer solution that was used for determining the Br uptake. Despite selection of an albumin with low Cl content, the standard contained a substantial amount of Cl (2–3 mM) which had to be taken into account by correcting the Br uptake accordingly. Blank values for Br were determined from skin pieces that were incubated with a Br-free standard solution. Cell volume (nl/cm^2) was estimated from the cross-sectional area in freeze-dried sections per section length. Br influx was calculated by multiplying the Br uptake (mmol/liter/min) with the cell volume.

Values given in the text are mean ± SD, with the number of observations in parentheses. Student's *t*-test was applied to determine differences between mean values. Unless stated differently, data are from nuclear measurements only.

Results

The experiments were performed on a batch of frogs that spontaneously exhibited a high transepithelial conductance, with control values for g_t of >1.0 mS/cm^2. Compared to the intact skin, the isolated epithelium had somewhat higher g_t values, suggesting that splitting of the skin increases the conductance. However, g_t was inhibitable by amiloride (*see* Fig. 5), ruling out any gross mechanical damage of the epithelium.

Mitochondria-rich Cells

In plots of the Br *vs.* the anion concentration (Br + Cl), two different groups of MR cells can be distinguished (Figs. 1, 3, and 6): cells close to the line of identity in which most of the intracellular Cl has been exchanged with Br from the outer bath, and cells in the lower right corner of the graph, with high anion and low Br concentrations. Skins with higher g_t generally had more of the former type (active cells), whereas skins with lower g_t had more of the latter type (inactive cells). The number of active MR cells (Cl < 18 mmol/kg w.w.) was almost twice as high (252 out of 383 = 65.7%) as the number of inactive cells.

Figure 1 depicts an experiment in which pieces of the same skin had significantly different g_t values. In the piece with the highest g_t value (2.73 mS/cm^2), 76% of all MR cells were active, whereas in the piece with the lowest g_t (1.07 mS/cm^2) only 40% belonged to that group. A third skin piece with intermediate g_t (1.73 mS/cm^2) had 58% active cells. As shown on the right side of Fig. 1, the Na concentration of active cells was higher than in inactive cells. The Na and Br concentrations of active MR cells were positively correlated (R^2 = 0.621), i.e., cells with high Na also took up more Br. The relationship appeared to be nonlinear, with the Br uptake tending to saturate with increasing Na.

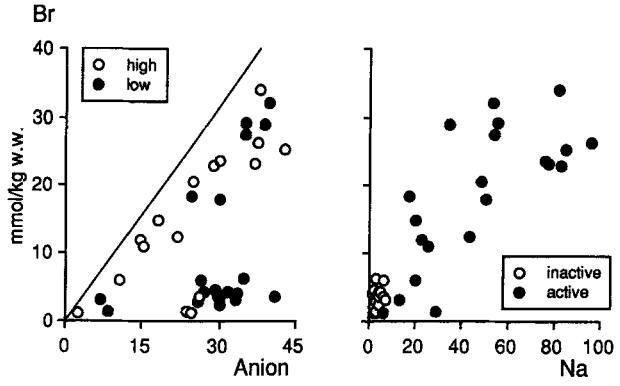

Fig. 1. Br uptake in skin pieces with spontaneously high and low g_t. On the left, the Br concentration of MR cells is plotted against the anion concentration (Br + Cl), on the right, against the Na concentration. For definition of inactive and active cells, *see text.*

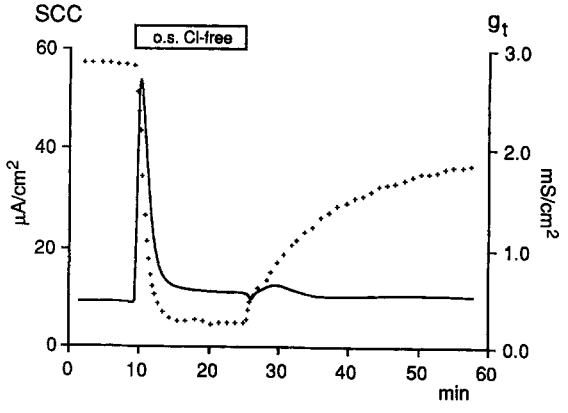

Fig. 2. Time course of the SCC (continuous line) and g_t following the exchange of the apical bath (outer solution) with a Cl-free Ringer solution (Cl replaced by SO_4).

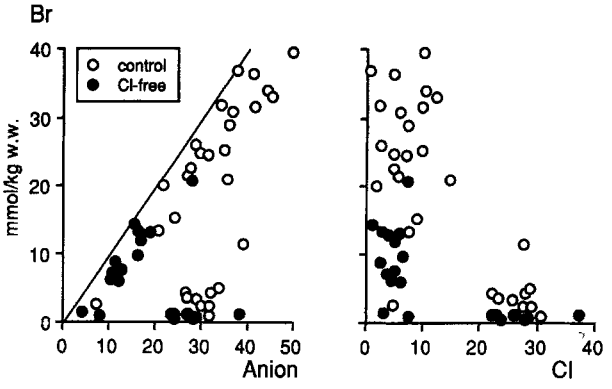

Fig. 3. Br uptake in control and after replacing the apical bath with a Cl-free solution. On the left, the Br concentration of MR cells is plotted against the anion concentration, on the right, against the Cl concentration.

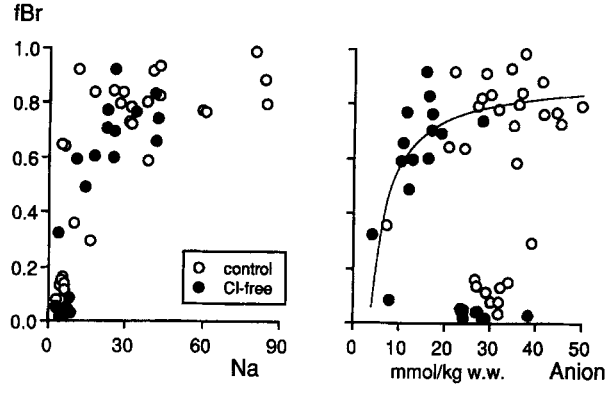

Fig. 4. Fractional Br uptake (fBr = Br/Anion) as a function of the Na concentration, left, and anion concentration, right. Same experiment as in Fig. 3. On the left, only one of the 19 MR cells in the cluster of low Na, low fBr cells is active. The curve on the right follows the relationship: fBr = 0.9–3.2/Anions.

Figure 2 illustrates one of the two experimental procedures that were used to decrease g_t. After replacing the outer bath with a Cl-free solution, g_t falls to very low values, indicating that g_t is largely due to a Cl conductance. The fall in g_t is paralleled by a large increase in SCC which can be explained by an efflux of Cl, driven by the large transepithelial Cl gradient. The SCC stimulation is transient, however, suggesting that the Cl conductance is switched-off during this time. After changing back to normal Ringer solution, the g_t does not immediately return to its original value[1]. Moreover, as previously noted in *Rana esculenta* (Rick, Dörge & Sesselmann, 1988), the SCC remained slightly elevated.

The effect of this experimental maneuver on the apical Br uptake is evident from Fig. 3. Compared to control, the Br concentrations of active and inactive cells are significantly reduced. In contrast, the Cl concentration remained nearly unchanged (right side of Fig. 3). Removal of Cl also resulted in a lower fractional Br uptake (fBr) and, limited to active cells, lower Na values (Fig. 4). In active MR cells, the decline in fBr appeared to be a nonlinear function of the reduction in the anion concentration (right side of Fig. 4).

Figure 5 shows an experiment in which the Na channel blocker amiloride was added to the outer bath.

[1] In part, the Cl conductance seems to be permanently switched off as, even after 60 to 90 min, the g_t value remained below control. The slow recovery of g_t cannot be explained by an incomplete washout of the Cl-free solution since (i) the exchange time of the chamber was ≤10 sec and (ii) low concentration of SO_4 had no effect on g_t.

While amiloride almost instantaneously blocked the SCC, limited only by the speed of the bath exchange, the decline of the g_t was relatively slow. The fast initial drop in g_t can be attributed to the inhibition of the apical Na channel, whereas the slow secondary decrease reflects the inactivation of the Cl permeability (Kristensen, 1983). With amiloride present, removal of Cl resulted in only a minute SCC peak (*not shown*), demonstrating that the Cl conductance is already switched-off. Subsequent clamping of the transepithelial voltage to 100 mV (inside positive) increased g_t from 0.17 to 0.72 mS/cm^2 which was less than the control value of 1.89 mS/cm^2 observed before addition of amiloride.

As evident from the left side of Fig. 6, amiloride blocked the Br uptake of active MR cells while the Br uptake of inactive cells remained virtually unchanged. Voltage clamping to +100 mV, in the continued presence of amiloride, led to a recovery of the Br uptake in some, but not all active cells (right side of Fig. 6). Voltage clamping had no effect on the Br uptake of inactive cells. Amiloride decreased the Na concentration of active MR cells from 47.5 ± 16.9 (n = 17) to 7.5 ± 2.4 mmol/kg w.w. (n = 16; 2P < 0.001), a result which was only partially reversible by voltage clamping (9.8 ± 2.8 mmol/kg w.w.; 2P < 0.01).

Table 1 summarizes the results of the Cl-free and amiloride experiments. Switching-off the Cl conductance by removing Cl or adding amiloride to the outer bath produced a large fall in the Na and Br concentrations of active MR cells. After amiloride, the Na value was almost as low as in inactive cells and the Br value was even lower. The decrease in the Na concentration was accompanied by an equivalent increase in the K concentration, indicating an exchange of Na for K. In contrast, the Na, Br, and K concentrations of inactive cells remained virtually unchanged, except for a small but significant reduction in the Br uptake after Cl-free incubation.

Active MR cells had much higher Na and Br values than inactive cells. However, this distinction was lost after amiloride. A criterion that was preserved under all experimental conditions is the lower Cl concentration of active cells. The slightly lower control values for P and Mg suggest that active cells have a higher water content. This notion is further supported by a lower dry weight fraction (21.4 ± 5.4 *vs.* 22.8 ± 3.9 g/100 ml; NS).

The anion concentrations of active MR cells in control and Cl-free revealed a bimodal frequency distribution, with cells in the switched-off (<15 mmol/kg w.w.) and switched-on state having mean values of 9.1 ± 3.7 (n = 28) and 31.3 ± 10.5 mmol/kg w.w. (n = 104; 2P < 0.0001), respectively. Off cells had a lower Na concentration compared to on cells (18.1 ± 17.2 *vs.* 57.7 ± 29.6 mmol/kg w.w.; 2P < 0.001) and, judging by the increased dry weight fraction, a smaller cell volume

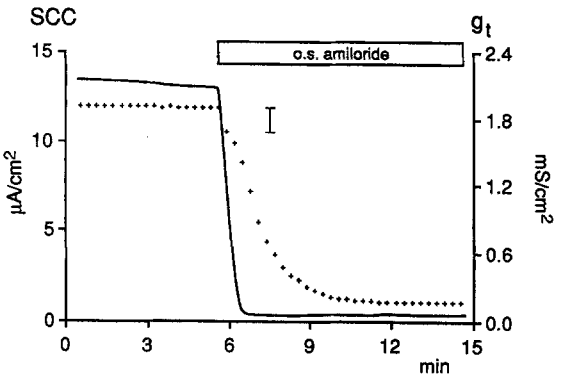

Fig. 5. Time course of SCC and g_t following the addition of amiloride (0.1 mM) to the apical bath. The I-beam indicates the fast component of the g_t decrease, reflecting the inhibition of Na transport.

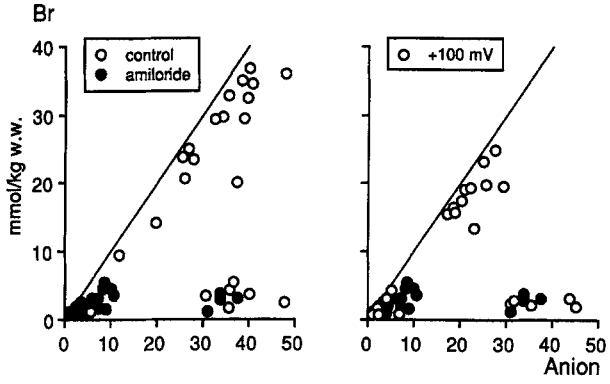

Fig. 6. Br uptake in control, after apical amiloride, and subsequent voltage clamping to 100 mV (inside positive). The Br concentration of MR cells is plotted as a function of the anion concentration. To facilitate comparisons, the data for amiloride are shown in both graphs.

(by 14.6 ± 10.9%; 2P < 0.002). After amiloride, all active cells were in the switched-off state. As evident from Table 1, the Na and anion concentration in this group were even lower than in other switched-off cells.

Table 1, as well as most of the figure, are based on nuclear measurements only. The cytoplasm of MR cells showed similar element concentrations, except for some systematic differences between nucleus and cytoplasm that were detectable also in principal cells (v.i.). Figure 7 compares the Br uptake in the nucleus and cytoplasm of active and inactive cells. Although for both cell types the Br concentration in the cytoplasm is higher than in the nucleus, the cytoplasmic fBr is significantly lower. This apparent contradiction can be explained by the higher anion concentration of the cytoplasm (lower left panel of Fig. 7). A particularly large gradient was observed for the Cl concentration of active cells, with Cl in the cytoplasm more than twice as high as in the nucleus (lower right panel of Fig. 7).

In control, the total anion concentrations of active

Table 1. Element concentrations of active and inactive mitochondria-rich cells in control, after apical incubation with a Cl-free solution, and after addition of amiloride

	n	Na	Mg	P	Cl	K	Br	fBr
Active Cells								
Control	89	58.6 ± 29.5	7.4 ± 2.1	121.9 ± 26.5	6.7 ± 3.9	73.1 ± 15.9	24.8 ± 9.3	0.763 ± 0.145
Cl-free	43	30.4[a] ± 12.6	10.1[a] ± 1.7	128.9 ± 33.4	3.8[a] ± 2.0	87.6[a] ± 21.4	10.4[a] ± 5.0	0.694[a] ± 0.181
Amiloride	31	7.3[a] ± 2.6	8.8[a] ± 2.1	129.7[a] ± 16.9	3.5[a] ± 2.1	124.7[a] ± 26.0	2.0[a] ± 1.5	0.466[a] ± 0.366
Inactive Cells								
Control	36	4.8[b] ± 1.6	10.2[b] ± 2.2	143.8[b] ± 35.2	24.4[b] ± 3.7	128.4[b] ± 30.7	3.2[b] ± 1.7	0.118[b] ± 0.063
Cl-free	31	5.4[b] ± 1.8	9.7 ± 1.3	137.8 ± 27.2	31.2[ab] ± 11.5	123.0[b] ± 24.3	1.0[ab] ± 0.5	0.030[ab] ± 0.016
Amiloride	12	5.5[b] ± 2.0	7.1[ab] ± 1.1	104.8[ab] ± 21.9	35.1[ab] ± 5.1	122.5 ± 20.4	3.1[b] ± 0.9	0.083[ab] ± 0.022

Mean ± SD of *n* nuclear measurements (mmol/kg wet weight). [a] Significantly different from control; [b] Significantly different from active cells ($2P < 0.05$). Data from five Cl-free experiments and two amiloride experiments; the controls for both protocols were combined.

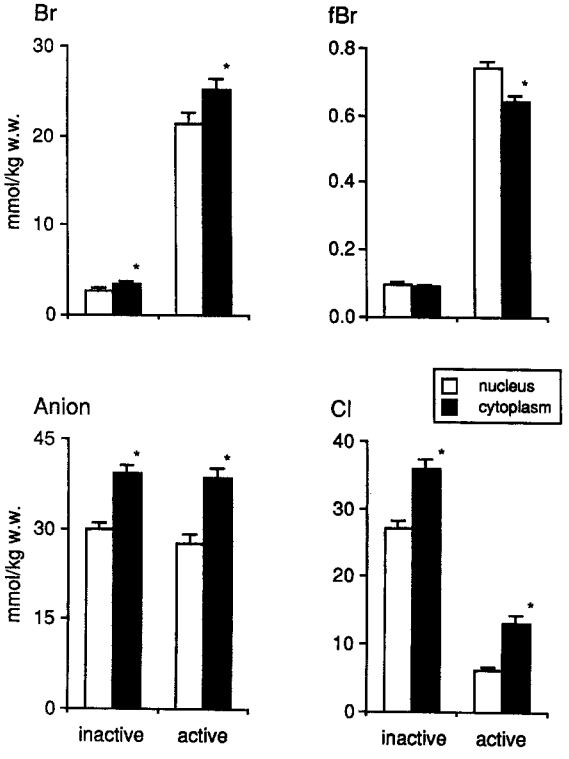

Fig. 7. Br concentration, fBr, total anion and Cl concentration in nucleus and cytoplasm of inactive and active MR cells (control skin). Mean values ± SE. *Values that are significantly different from the nucleus ($2P < 0.05$).

and inactive cells were very similar (*cf.* Fig. 7, Table 1). Thus, under conditions when both sides of the skin are bathed in normal Ringer solution, it will not be possible to distinguish between the two cell types on the basis of the Cl concentration alone. As an additional criterion, the Na concentration may be used which in active MR cells with high anions was invariably high (right side of Fig. 1).

Principal Cells

Table 2 lists the element concentrations of principal cells. Removal of Cl resulted in no significant variations in the nuclear concentrations of Na, Cl, and Br. In contrast, apical amiloride lowered the Na and Br concentration and raised the Cl concentration. Voltage clamping, with amiloride present, had no effect on Na and Cl, but significantly increased the nuclear Br value (to 1.1 ± 0.9 mmol/kg w.w.; $n = 61$; $2P < 0.006$). Amiloride and, to a lesser extent, also Cl-free reduced the Mg concentration.

The epithelial cell layers showed an inwardly directed Na concentration gradient that was slightly reduced after Cl removal and completely abolished after amiloride (Fig. 8). In contrast, the profile for Cl was essentially flat (right side of Fig. 8). In some of the skins, a small, inwardly directed Br and fBr gradient was detectable that was markedly enhanced by amiloride (Fig. 9). The Br concentration in deeper layers was often not significantly different from the blank value (0.2–0.3 mM) for Br.

As reported previously (Rick et al., 1984), the cytoplasmic Na, Mg, and Cl concentrations were slightly higher than in the nucleus, whereas the P value was significantly lower (Table 2). Similar concentration differences between nucleus and cytoplasm were observed after Cl-free incubation and amiloride. In addition, the cytoplasmic Br value always exceeded the nuclear value.

Discussion

Based on the short-term Br uptake, two types of MR cells can be distinguished: cells that rapidly exchange with the outer bath and cells that do not. The first cell was called active as its Br uptake and frequency were positively correlated with g_t, whereas for inactive cells

Table 2. Element concentrations in nucleus and cytoplasm of principal cells in control, after apical incubation with a Cl-free solution, and after addition of amiloride

	n	Na	Mg	P	Cl	K	Br	fBr
Nucleus								
Control	310	10.2 ± 4.3	8.3 ± 1.5	109.4 ± 27.4	37.5 ± 5.4	125.1 ± 31.8	1.0 ± 0.8	0.028 ± 0.023
Cl-free	256	10.3 ± 4.4	7.8[a] ± 1.6	106.9 ± 23.1	37.0 ± 7.6	126.1 ± 29.6	0.9 ± 0.7	0.026 ± 0.020
Amiloride	56	5.1[a] ± 2.3	6.4[a] ± 1.6	105.9 ± 23.6	43.6[a] ± 5.0	126.2 ± 28.1	0.6[a] ± 0.9	0.013[a] ± 0.021
Cytoplasm								
Control	341	12.0[b] ± 5.9	9.2[b] ± 2.1	62.7[b] ± 24.4	48.9[b] ± 6.6	121.7 ± 37.2	1.5[b] ± 1.4	0.030 ± 0.027
Cl-free	352	11.1[a] ± 5.8	8.3[ab] ± 2.0	60.9[b] ± 23.7	48.7[b] ± 8.9	123.3 ± 37.2	1.1[ab] ± 1.2	0.022[ab] ± 0.022
Amiloride	54	5.5[a] ± 2.8	8.5[a] ± 2.0	71.1[ab] ± 24.9	56.4[ab] ± 5.1	122.9 ± 29.6	1.1[ab] ± 1.2	0.019[a] ± 0.020

Mean ± SD of *n* measurements (mmol/kg wet weight). [a] Significantly different from control; [b] Significantly different from the nucleus ($2P < 0.05$). Data from five Cl-free experiments and two amiloride experiments; the controls for both protocols were combined.

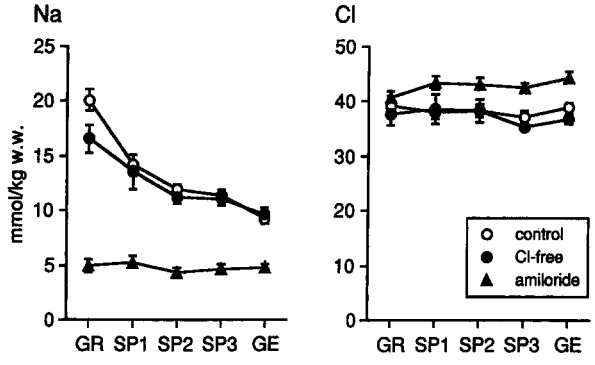

Fig. 8. Na and Cl concentrations of principal cells in control and after replacing the apical bath with a Cl-free solution. In addition, amiloride data of an experiment with matching control are shown. The different epithelial layers are (from apical to basal): stratum granulosum (*GR*), spinosum (*SP1, SP2, SP3*) and germinativum (*GE*). Mean values ± SE.

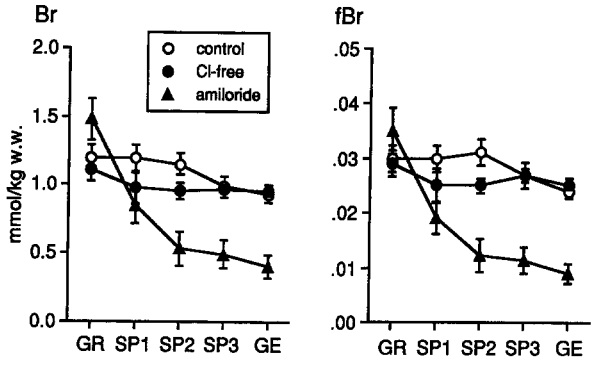

Fig. 9. Br concentrations and fBr of principal cells. Same experiments as in Fig. 8.

this was not the case. The Na and Br concentrations of active cells were much higher than in inactive cells; however, the distinction was lost after switching-off the Cl conductance by amiloride. A more reliable indicator of an active MR cells is the lower Cl concentration, i.e., the amount of intracellular anions not exchangeable by apical Br (right side of Fig. 3; Table 1). Active cells appeared to be slightly swollen compared to inactive cells. The difference was not large enough to allow identification of the MR cell type in freeze-dried sections by size or electron-optical density.

Figure 10 depicts the Br concentrations of MR and principal cells as a function of the simultaneously measured g_t. It is evident that the g_t is correlated with the Br uptake of MR cells ($R^2 = 0.878$; $P < 0.0001$), but not of principal cells ($R^2 = 0.012$; NS). The correlation is largely due to changes in the Br uptake of active cells (Table 1). In addition, a variable proportion of active and inactive cells may account for some of the g_t differences among controls (Fig. 1). A third factor, which because of the experimental design played only a minor role, is the total number of MR cells (Vôute & Meier, 1978)[2].

The Br uptake of MR cells is equivalent to a Br influx of 2.2 nmol/min/cm^2, from which, using the constant-field equation, a Cl permeability of 9.3 nm/sec and a conductance 0.14 mS/cm^2 can be calculated[3]. This value is about one order of magnitude lower than the g_t value in control (1.82 mS/cm^2) and, thus, appears to be too small to fully account for the Cl conductance of the skin. On the other hand, the initial Br influx is presumably much larger than the 1 min uptake obtained here. In skins in which the Br uptake was followed for 60 min (R. Rick, *unpublished results*), active cells had

[2] Comparisons were made between symmetrical skin pieces obtained from the same frog that were, in addition, closely matched with regard to g_t.

[3] The apical membrane potential was set at −40 mV. Assuming that Br and Cl in active cells are passively distributed, the potential of switched-on and switched-off cells can be estimated from the anion concentration to be −27 and −59 mV, respectively.

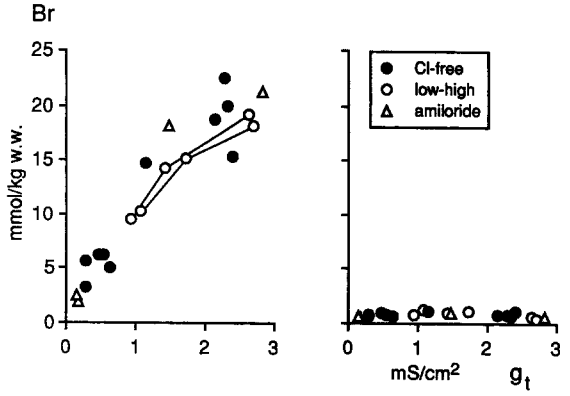

Fig. 10. Br uptake of MR cells, left, and principal cells, right, as a function of g_t. Control skins with spontaneously low, medium, and high g_t are connected by lines. Symbols denote type of experiment, not the actual experimental condition. Data for MR cells comprise active and inactive cells.

an average fBr of 0.737 ± 0.106 (n = 33), suggesting that already after 1 min the anion exchange was complete. To explain the Cl conductance by active MR cells alone, the half-time of the Br exchange has to be 8 sec, a value within the expected range. For inactive MR cells, the fBr after 60 min was 0.182 ± 0.038 (n = 28), consistent with a half-time of about 40 sec.

As indicated by the control values (low-high) in Fig. 10, the relationship between the Br uptake of MR cells and g_t is slightly curved. This impression is further supported by the fact that the data without amiloride should have a non-zero x-intercept, representing the conductance of the Na transport pathway. A plausible explanation for this nonlinearity is that switching-off the Cl conductance increases the half-time of the anion exchange. Thus, the uptake at low g_t should be a better approximation of the initial Br influx than the uptake at high g_t. A slower Br exchange must be anticipated if the anion permeability drops faster than the anion content of the cell, which is likely to be the case.

The study did not address the question of by which mechanism the Cl conductance of the apical membrane is switched-on or off. However, the complete inhibition of the Br uptake by amiloride and its partial recovery by voltage clamping is consistent with a voltage-gaged Cl channel in the apical membrane (Larsen, 1991). Activation of the channel by voltage clamping, with amiloride present in the apical bath, resulted in only a minimal increase of the Na concentration, demonstrating that the Na influx is not absolutely required. Nevertheless, Na may play a facilitating role as indicated by the finding that not all active MR cells were switched-on and the Br influx was less than in control (Fig. 6). The low Na concentration of active cells after amiloride implies that the apical, amiloride-sensitive Na channel is the only major leak pathway. The presence of an apical Na channel in MR cells has been recently confirmed by the patch-clamp technique (Harvey, 1992).

The low Br uptake and the lack of a response to amiloride and voltage clamping argues against a significant involvement of inactive MR cells in passive Cl or active Na transport. In fact, inactive cells accounted for only 4.2% of the Br uptake of MR cells, or even less when factoring in the much slower exchange. Since the frog skin is capable of secreting hydrogen ions (Ehrenfeld & Garcia-Romeu, 1977), it is possible that this cell type corresponds to acid-secreting cells observed in other urinary epithelia (Steinmetz, 1986). The presence of a subpopulation of cells that is not substantially contributing to the Cl conductance is also supported by an investigation in *R. pipiens* in which a current peak was detectable by a vibrating probe over only about 50% of all MR cells (Foskett & Ussing, 1986).

We recently reported that amiloride lowered the Na and Cl concentration of some, but not all MR cells (Rick, 1992). Also, some of the cells experienced a large Na increase after ouabain that was inhibitable by amiloride. The present data suggest that it is the active MR cell which is sensitive to both amiloride and ouabain. Presumably, this cell is equivalent to the amiloride- and ouabain-sensitive MR cell identified in the toad skin epithelium (Larsen et al., 1987). Since its transport characteristics are distinct from α- and β-cells in the turtle urinary bladder, this cell type has been named the γ-cell (Larsen, 1991).

Larsen et al. (1987) pointed out that the MR cell participates in active Na transport, although because of the low number of cells, their contribution is probably very small. This raises the possibility that the slight stimulation of the SCC seen after Cl removal (Fig. 2) can be explained by an increased Na influx through MR cells. However, the fact that Cl removal decreased the Na concentration of active cells argues against this possibility. Perhaps, a more likely explanation is the inhibition of active Cl reabsorption, a transport process that has been described for skins of low potential of the southern variety of *R. pipiens* (Watlington & Sessee, 1973) and also resides in the MR cell (Larsen, 1991).

Active cells exist in two functional states: switched-on and off. In control and after Cl removal, switched-off cells had a 15% smaller cell volume than on cells. A similar degree of shrinkage was detectable after amiloride. The volume change is nearly identical to that observed for MR cells in the toad (Larsen et al., 1987), but slightly less than in *R. pipiens* (Spring & Ussing, 1986). Shrinkage was accompanied by a large fall in the Na and anion concentration, suggesting that the water loss is caused by an efflux of NaCl (NaBr). Apparently, volume changes *per se* are not responsible for activation or inactivation of the Cl conductance (Larsen et al., 1987).

Amiloride had no effect on the Na and Br concentrations of inactive MR cells. Moreover, the proportion of inactive cells agreed well with the proportion of cells that did not respond to ouabain (Rick, 1992), indicating that this cell is insensitive to both ouabain and amiloride. The lack of a Na increase after ouabain does not necessarily imply that inactive cells do not possess a Na pump as the Na leak could be so small that the residual pump activity is able to maintain a low Na concentration. That inactive cells had extremely low Na values, even lower than active cells after amiloride, provides some support for this view. The high Cl values point to the presence of a transport mechanism that is able to raise Cl above electrochemical equilibrium.

In the intact skin of *Rana temporaria* and *esculenta* (Rick et al., 1978), MR cells with very low Na and Cl concentrations were observed that were insensitive to amiloride and only moderately sensitive to ouabain. In light of the present results, these cells can be classified as active, switched-off cells. The reduced response to ouabain may be due to inactivation of the amiloride-sensitive Na channel in the switched-off state. In contrast, most cells in the present study appeared to be in the switched-on, Cl-conducting state, consistent with the high g_t value of the isolated epithelium of *R. pipiens*. Splitting of the skin increased g_t, suggesting that the mechanical stress experienced during separation of the epithelium or the exposure to collagenase leads to activation of the Cl conductance.

In skins of *Bufo viridis* (Dörge et al., 1988), voltage clamping increased for fBr of MR cells from 0.26 to 0.44, supporting the view that the MR cell is involved in voltage-activated Cl conductance. However, no correlation was detectable between the Br influx and g_t. This may be explained by (i) the small number of MR cells analyzed, (ii) the long incubation time (15–30 min), (iii) the failure to distinguish between active and inactive MR cells, and (iv) the low g_t value of the intact skin. In fact, based on the criteria applied here, only 9 out of 25 active MR cells (36%) in control were switched-on, whereas in the present study the percentage was considerably higher (119 out of 154 = 77%).

Within 1 min, more than 76% of the Cl of active MR cells was replaced by apical Br and, even after switching-off the Cl conductance by amiloride, almost half of the intracellular Cl was exchangeable from the outside (Table 1). This result appears to be in conflict with a computer simulation of the MR cell which predicts a significantly lower Cl permeability of the apical membrane compared to the basolateral membrane (Larsen & Rasmussen, 1985). One has to keep in mind, however, that the estimation of the Cl conductance is predicated on the unproven assumption that the Br influx is electrodiffusive and the pathways involved have the same affinities for the two halide ions. In the toad skin, the voltage-activated Cl current displays a poor anion selectively, with a slight preference of Br over Cl (Harck & Larsen, 1986). Yet fully activated, the Br current was somewhat less than the Cl current.

Due to the large number of cells, principal cells took up more Br than MR cells. The Br uptake in control was equivalent to a Br influx of 3.4 nmol/min/cm^2 or, after subtracting the Br blank value, 2.6 nmol/min/cm^2. The influx was not correlated with g_t, consistent with an electroneutral uptake mechanism in the apical membrane (Biber et al., 1985; Drewnowska & Biber, 1985) and lack of a significant Cl conductance (Willumsen & Larsen, 1986). As in the intact skin (Dörge et al., 1985), the Cl of principal cells was largely exchangeable from the basolateral side, with a half-time of about 30 min (R. Rick, *unpublished results*). This and the presence of narrow exit slits which may limit the diffusional exchange with the inside (Rick, 1992), raises the possibility that some Br is taken up from the spaces between the cells. Consequently, the results for principal cells should be considered with some circumspection.

The effects of amiloride on the Na concentration support the view that the principal cell is primarily involved in active Na reabsorption. In addition, amiloride increased the Cl concentration, presumably by enhancing the driving force for the basolateral cotransporter (Rick, 1992). The way by which amiloride lowers the Mg concentration of principal cells is unknown; one possibility is an Na/Mg exchange mechanism, similar to that described for frog skeletal muscle (Blatter, 1991).

In active MR cells, the nuclear fBr exceeded the cytoplasmic value, suggesting that the nucleus exchanges directly with the outer bath and not indirectly, via the cytoplasm. Also, the amount of Cl that was not exchangeable by apical Br was about twice as high as in the nucleus (lower right panel of Fig. 7). Perhaps, a more likely explanation for the low cytoplasmic fBr is that the cytoplasm is compartmentalized, with a large fraction of the Cl located in a poorly exchangeable subspace. For inactive MR cells and principal cells, the nuclear and cytoplasmic fBr values were not statistically different. However, this may be simply due to an inability to demonstrate such a difference at the very low Br levels found in these cells.

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