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Intracellular Ion Concentrations in the Isolated Frog Skin Epithelium: Evidence for Different Types of Mitochondria-Rich Cells

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Summary. Intracellular ion concentrations were determined in split skins of Rana pipiens using the technique of electron microprobe analysis. Under control conditions, principal cells and mitochondria-rich cells (MR cells) had a similar intracellular ion composition, only the Cl concentration in MR cells was significantly lower. Inhibition of transepithelial Na transport by low concentrations of ouabain (2×10^{-6} M, inner bath) resulted in a Na concentration increase of principal cells from 10.9 to 54.3 mmol/kg wet wt. The increase was completely abolished by simultaneous application of amiloride (10^{-4} M, outer bath). Amiloride alone resulted in a significant decrease of the Na concentration to 6.1 mmol/kg. w.w. Among MR cells, two different groups of cells could be distinguished; cells that showed a Na increase after ouabain which was even larger than that in principal cells and cells that did not respond to ouabain. In about half of all ouabain-sensitive MR cells the Na increase could be prevented by amiloride. According to these results, a subpopulation of MR cells displays the transport characteristics expected for a transepithelial Na transport compartment, an apical amiloride-sensitive Na influx and a basal ouabain-inhibitable Na efflux. Given the small number of cells, however, it is unlikely that this subtype of MR cells contributes significantly to the overall rate of transepithelial Na transport.

Key Words intracellular ion concentration · mitochondria-rich cell · principal cell · x-ray microanalysis · transepithelial Na transport · ouabain · amiloride.

Introduction

In an earlier study using electron microprobe analysis to measure the intracellular ion concentrations, we have shown that all layers of the multilayered frog skin epithelium participate in transepithelial Na transport, forming a syncytial Na transport compartment (Rick et al., 1978). Cornified cells, gland cells, and mitochondria-rich cells (MR cells) were found to be no part of the transport syncytium. The syncytial organization of the epithelium was also supported by impedance analyses (Smith, 1971), intracellular potential measurements (Nagel, 1976), and ouabain-binding studies (Mills, Ernst & DiBona, 1977).

Recently, some evidence has been provided that the MR cell constitutes a parallel pathway for transepithelial Na transport. From the initial rate of cell volume change after amiloride, the Na transport activity of MR cells in the toad skin epithelium was estimated to be even higher than that of principal cells (Larsen, Ussing & Spring, 1987). This finding is in conflict with our earlier observation that the Na influx in MR cells cannot be blocked by amiloride (Rick et al., 1978). Additional support for an important role of MR cells in transepithelial Na transport comes from the observation of a parallel increase in MR cell number and transport rate after adaptation of the frogs to Na-free solutions (Ehrenfeld, Lacoste & Harvey, 1989).

To evaluate the possible role of MR cells in transepithelial Na transport, we determined the intracellular ion concentrations in the isolated frog skin epithelium of *Rana pipiens*. The measurements were performed on freeze-dried cryosections using electron microprobe analysis. Changes in the ion concentrations were followed after blocking the apical Na influx by amiloride and after inhibition of the basolateral Na efflux by ouabain. According to the results, different subpopulations of MR cells can be distinguished, only one of them displaying the behavior of a Na transport compartment. The contribution of this cell type to the overall rate of Na transport is likely to be small.

Materials and Methods

Frogs of the northern variety of *Rana pipiens* were obtained from Lemberger (Oshkosh, WI) and kept at room temperature (22°C) in plastic troughs with free access to tap water. Water was exchanged daily. Frogs were sacrificed by doubly pithing. The abdominal skin was dissected and cut into four pieces which were glued with cyanoacrylate glue onto thin rings made out of electroformed nickel. The pieces were then incubated for about 90 min in Ringer's solution containing 1 mg/ml type 2 crude collagenase

(Worthington Biochemicals, Freehold, NJ) until the rings with the epithelium attached could be stripped off the underlying connective tissue (Fisher, Erlij & Helman, 1980).

After isolation, the epithelia were mounted in Ussing-type incubation chambers for recording of open-circuit potential and short-circuit current (I_{sc}). Small signal conductance (g_t) was monitored by clamping the transepithelial potential for 500 msec to ±10 mV. Initially all four pieces were incubated in Ringer's solution for about 60 min until the I_{sc} had reached a steady-state value (control-I_{sc}). Then, ouabain (inner bath, 2×10^{-6} M), amiloride (outer bath, 10^{-4} M), or a combination of both inhibitors were applied for 90 min. One piece remained untreated, serving as control.

Ringer's solution contained (in mM): 110 NaCl, 2.5 $KHCO_3$, 1 $CaCl_2$, and 5 glucose. Ouabain (Sigma, St. Louis, MO) and amiloride (a gift from Merck, Sharp & Dohme, Rahway, NJ) were applied from highly concentrated stock solutions. All media were equilibrated with air and had a pH of between 8.0 and 8.3. Albumin standards were prepared by dissolving 20 g% lyophilized bovine serum albumin (Sigma, St. Louis, MO) in Ringer's solution, adding 0.14 ml water/ml to offset the resulting increase in osmolarity.

At the end of the incubation, the epithelia were quickly removed from the chamber and the mucosal surface was coated with a thin layer of the albumin standard solution. The tissue was shock-frozen by plunging the rings sideways into melting ethane (−188°C) using a catapult-like device. Less than 5 sec elapsed between layering with the standard solution and freezing. From the frozen material sections of about 250 nm thickness were cut dry with a glass knife at −140°C in a cryoultramicrotome (Reichert FC4 D, Vienna, Austria). The sections were mounted on parlodion films and dried overnight at −90°C and 10^{-7} mbar.

Microanalysis of the sections was performed in a scanning electron microscope (Stereoscan S250, Cambridge Instruments, Cambridge, U.K.) which was equipped with a solid-state x-ray detecting system (Link AN10000, High Wycombe, U.K.) and a special transmission stage for x-ray microanalysis. Typical measuring conditions were 20 kV acceleration voltage, 0.5 nA probe current (continuously monitored during analysis), and 100 sec analysis time. The emitted x-rays were recorded in the energy range from 0 to 10 keV, containing the $K\alpha$ lines of the light elements Na, Mg, P, S, Cl, K, and Ca. After film stripping, the intensities of the element-characteristic radiations and noncharacteristic background radiation were evaluated by a modification of a previously described computer program (Bauer & Rick, 1978). Quantification of the cellular element concentrations (mmol/kg wet wt) and dry weight content (g dry matter/100 ml cell) was achieved by a comparison of the cell x-ray spectra with those obtained in the adherent albumin standard layer (Rick, Dörge & Thurau, 1982).

The values are expressed as means ± SD (standard deviation). Student's *t*-test was applied to determine whether differences in the means attained statistical significance. Data points in figures are nuclear values only, except for the cell volume (*see* Fig. 6) which was calculated based on the average nuclear and cytoplasmic dry weight contents.

Results

EPITHELIAL MORPHOLOGY

Figure 1*A* shows a scanning transmission electron micrograph of a freeze-dried cryosection on which the analyses were performed. In addition to several layers of principal cells, the isolated epithelium contains an outer layer of cornified cells and several mitochondria-rich cells (MR cells). MR cells can be identified by the flask-like shape and the large number of mitochondria in the apical cytoplasm. In addition, this cell type often has a somewhat lighter or darker appearance of the cytoplasm compared to neighboring principal cells and does not share in the dense filamentous network which seems to pervade the whole epithelium (Rick, 1989). Frequently, the narrow apical neck region of the MR cell was not visible in the section in which the measurement was made. However, in most cases where the same cell could be followed in serial sections, a connection with the apical surface could be established. Typically, the apical pole of the MR cell was located underneath a junctional complex in the stratum corneum (arrow in Fig. 1*A*).

After ouabain and, less frequently, in control and after amiloride plus ouabain, MR cells were observed which were swollen, as suggested by the large cell size and low electron density. Occasionally, the spaces around these cells appeared to be filled with some matrix material (Fig. 1*B*). Since membranes are not stained in this preparation, it is possible that the material corresponds to a peripheral band of organelle-free cytoplasm.

The innermost cell layer often showed some evidence for cell damage, presumably due to the splitting of the skin. While in the intact skin basal cells typically are small and have a columnar shape, in the isolated epithelium basal cells often had an irregular shape like cells of the stratum spinosum (Fig. 1*A* and *C*). In about one third of all sections grossly swollen cells were detectable in the basal cell layer. Occasionally, these cells were found sloughed off to the inner bath, but still being attached to the epithelium (Fig. 1*A*). The intraepithelial portion of glandular ducts was present in some sections; the remainder of the duct was apparently torn off during splitting of the skin.

In control, the intercellular spaces of the epithelium were usually wide open, whereas after amiloride the spaces tended to be collapsed. Exit slits from the lateral intercellular spaces into the inner bathing medium were extremely narrow, often below the resolution afforded by the sections. Figure 1*C* shows a section in which the albumin standard, varying from the normal protocol, was applied to the inner side. It is evident that albumin does not penetrate into the lateral intercellular spaces during the 5 sec incubation time.

ELECTROPHYSIOLOGICAL PARAMETERS

Figure 2 illustrates the typical changes in the short-circuit current, I_{sc}. At the low concentrations of ouabain used in this study (2×10^{-6} M), net transepithe-

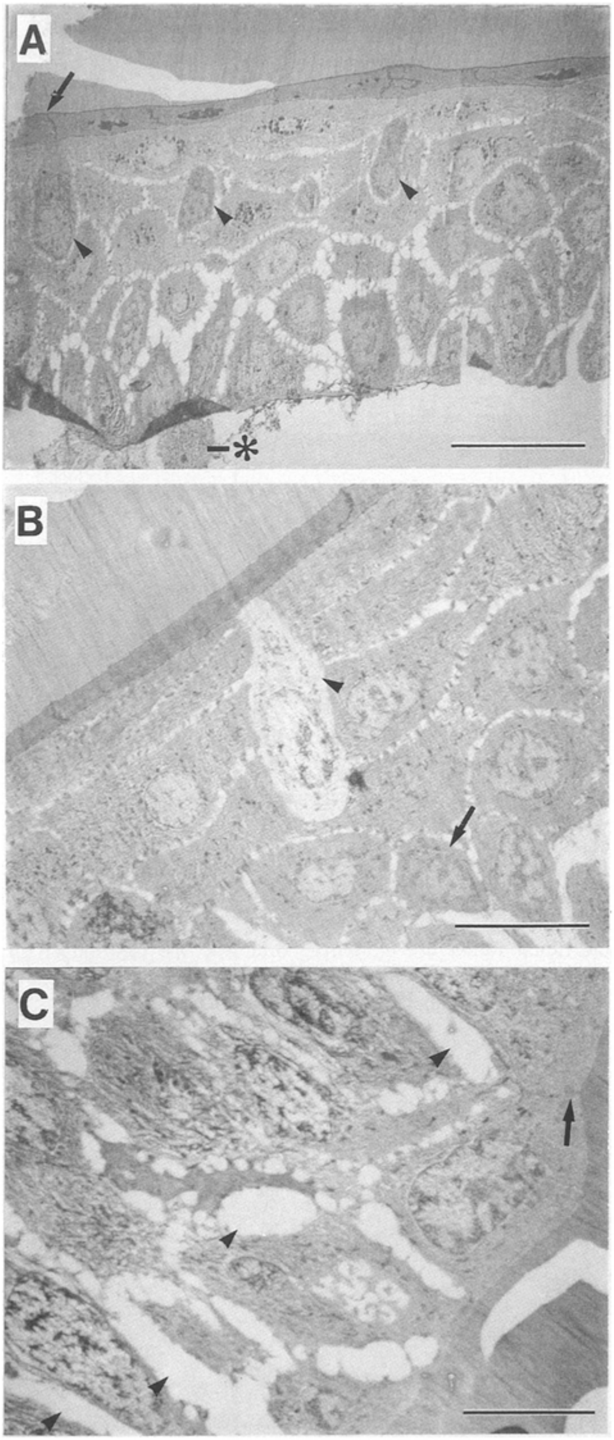

Fig. 1. Scanning transmission EM of a freeze-dried cryosection of an isolated frog skin epithelium (*Rana pipiens*). (*A*) The different cell layers are (from top to bottom): stratum corneum, granulosum, spinosum, and germinativum (basale). Between granulosum and spinosum a transitional cell layer with characteristics of both granular and spiny cells can be distinguished. The section contains three MR cells (arrowheads), only one of which shows a connection to the subcorneal space. The homogeneous layer on the top corresponds to the albumin standard used for quantification. The two dark structures on the lower left are caused by folding of the section. One sloughed-off cell (asterisk) and some cell detritus is visible below the epithelium. (*B*) Apical aspect of the epithelium. Note the presence of some matrix material in the lateral intercellular space around the swollen MR cell (arrowhead). The arrow points to a spinosum cell with low Cl concentration. (*C*) Basal aspect of the epithelium. The lateral intercellular spaces are wide open except for narrow exit slits to the serosal bath (arrow). Large gaps between cells (arrowheads) are caused by breaking up of desmosome connections and cell shrinkage during freeze drying. The size of the scale markers in *A, B* and *C* is 16, 8, and 5.5 μm, respectively.

lial Na transport as measured by the I_{sc} is not fully inhibited, even after 90 min of incubation. At the time of freezing, the I_{sc} averaged 29 ± 13% (n = 7) of the control-I_{sc}. After correcting for the slight decline in I_{sc} observed in the untreated control skin piece, the inhibition by ouabain amounted to 68.2%. In contrast, amiloride (10^{-4} M) almost completely abolished the I_{sc} (95.6 ± 3.2% inhibition). After amiloride and, to a lesser extent, after ouabain a significant reduction in the transepithelial conductance, g_t, was observed. The I_{sc} and g_t changes after simultaneous application of amiloride and ouabain were indistinguishable from those after amiloride alone.

Compared to a group of intact skins, the I_{sc} values of split skins were slightly lower and the g_t values were slightly higher. The time course of the I_{sc} and g_t changes were similar, except for the fact that the nonlinear decline of the I_{sc} after ouabain seen in the intact skin (Huf, Howell & Boswell, 1982) was absent in the isolated epithelium. Since the isolated epithelium does not contain glands, this finding suggests that the nonlinear decline is due to a glandular I_{sc} component. Intracellular ion concentrations reported below are from isolated epithelia only.

INTRACELLULAR ION CONCENTRATIONS

Figure 3 illustrates the changes in the Na concentrations of principal and MR cells in a typical experiment. Under all four experimental conditions, the scatter of the Na values of principal cells is relatively small, consistent with the view that the different layers of epithelium form a functional syncytium (Rick et al., 1978). After ouabain, the Na concentrations in all epithelial layers are increased. In contrast, after amiloride and after amiloride plus ouabain the Na concentrations are significantly reduced compared to control. In control and, to a lessor extent, after ouabain the Na concentrations in the outer epithelial cells are slightly higher than in the

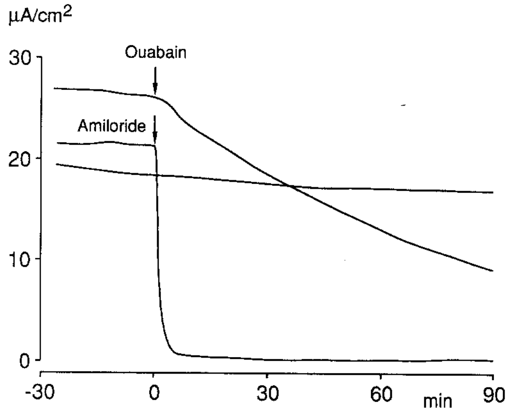

Fig. 2. Effect of ouabain and amiloride on short-circuit current, I_{sc}, of the isolated frog skin epithelium (*Rana pipiens*). Exposed skin area is 1.5 cm^2.

inner cells, creating the impression of an inwardly directed Na concentration gradient. The Na concentration in MR cells is highly variable, in particular after ouabain and after amiloride plus ouabain where some MR cells with very high Na concentration can be observed.

Cells in the basal layer that were obviously swollen or caught in the process of being sloughed off from the epithelium showed an almost extracellular-like composition of ions with high Na, high Cl, and low K concentrations. Consistent with their swollen appearance, the dry weight content and P concentration were markedly reduced. As shown in Fig. 4, the increase in the Na concentration was accompanied by a large gain in Ca and an almost complete loss of Mg. The accumulation of Ca in the nucleus was about twice as high as in the cytoplasm (*data not shown*), indicating that the nucleus contains significantly more binding sites for divalent cations.

Occasionally, spinosum cells were observed which differed from neighboring cells by having a much lower Cl concentration (arrow in Fig. 1*B*). Further measurements were made in glandular ducts. In favorable sections through the center of the duct, an outer and inner layer of cells could be distinguished. While the outer cells showed a Na increase after ouabain comparable to that in principal cells, in inner cells the Na concentration was virtually unchanged. The fact that the two layers can maintain large differences in the Na concentration suggests that they are not connected by low resistance cell-to-cell junctions.

Table 1 lists the average ion concentrations of principal cells, excluding swollen basal cells and the occasional low Cl cell. Despite a highly variable response, in all experiments a statistically significant Na increase was observed after ouabain. The in-

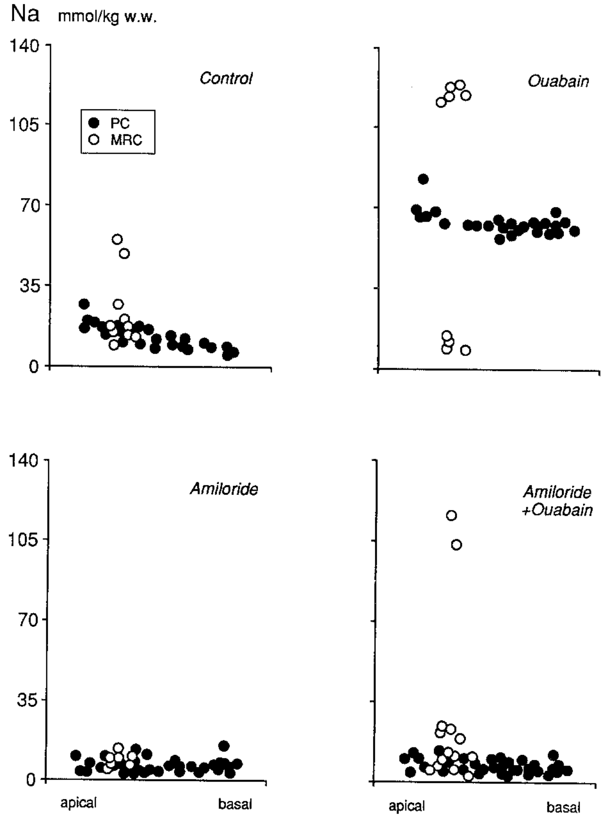

Fig. 3. Na concentration in principal cells (filled circles) and mitochondria-rich cells (open circles) of the isolated frog skin epithelium (*Rana pipiens*) in control, after ouabain, after amiloride, and after combined application of amiloride and ouabain. Individual values are plotted according to the position of the nucleus within the epithelium (on an apical-to-basal axis). Data from 3–4 sections per experimental condition.

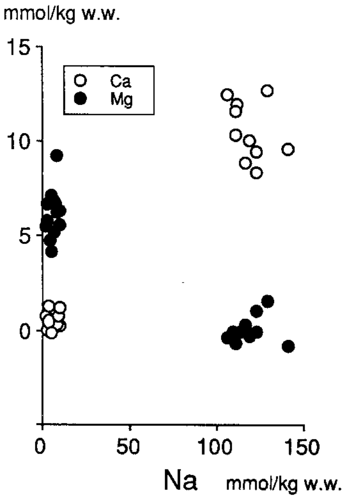

Fig. 4. Ca (open circles) and Mg concentration (filled circles) in basal cells as a function of the intracellular Na concentration. Data from two sections of a control epithelium. All cells with high Na concentrations (on the right) were visibly swollen.

Table 1. Ion concentrations and dry weight content of principal cells in the isolated frog skin epithelium (*Rana pipiens*)

		Na	K	Cl (mmol/kg wet wt)	Mg	Ca	P	Dry wt g%	
Nucleus	Control	10.9 ± 5.9	114.2 ± 17.1	34.6 ± 8.9	7.2 ± 2.0	0.8 ± 0.9	116.2 ± 33.5	22.1 ± 5.4	(366)
	Ouabain	54.3 ± 29.1^{b}	73.0 ± 26.6^{b}	26.7 ± 8.9^{b}	7.7 ± 2.1^{b}	0.7 ± 0.7	117.4 ± 36.0	24.9 ± 5.7^{b}	(382)
	Amiloride	6.1 ± 3.2^{bc}	129.2 ± 31.9^{bc}	38.6 ± 10.8^{bc}	7.0 ± 2.3^{c}	0.5 ± 0.8^{bc}	114.4 ± 48.6	22.0 ± 6.1^{c}	(218)
	Am + Ouab	8.0 ± 4.4^{bcd}	122.6 ± 26.5^{bcd}	34.9 ± 10.7^{cd}	7.0 ± 2.2^{c}	0.6 ± 0.8^{b}	111.3 ± 37.5	22.6 ± 9.6^{c}	(315)
Cytoplasm	Control	11.9 ± 7.1^{a}	114.7 ± 18.9	49.1 ± 11.4^{a}	8.2 ± 3.0^{a}	1.1 ± 1.2^{a}	68.4 ± 26.2^{a}	28.8 ± 9.5^{a}	(306)
	Ouabain	44.2 ± 26.9^{ab}	84.0 ± 31.5^{ab}	39.4 ± 11.8^{ab}	8.4 ± 2.7^{a}	1.3 ± 1.5^{a}	71.9 ± 28.6^{a}	32.8 ± 6.1^{ab}	(258)
	Amiloride	7.4 ± 5.6^{abc}	122.3 ± 26.9^{abc}	53.6 ± 12.5^{abc}	7.7 ± 2.4^{abc}	0.8 ± 0.8^{abc}	63.3 ± 26.1^{ac}	28.3 ± 6.5^{ac}	(134)
	Am + Ouab	9.3 ± 5.7^{abcd}	124.3 ± 23.5^{bc}	52.6 ± 11.8^{abc}	8.4 ± 3.0^{ad}	1.3 ± 1.1^{ad}	71.7 ± 34.4^{ad}	28.6 ± 8.9^{ac}	(260)

Mean values ± SD of (*n*) individual measurements in nucleus or organelle-free cytoplasm. Superscripts denote the statistical significance at $2P < 0.05$ level: [a]different from nucleus; [b]different from control; [c]different from ouabain; [d]different from amiloride.

Table 2. Ion concentrations and dry weight content of mitochondria-rich cells in the isolated frog skin epithelium (*Rana pipiens*)

		Na	K	Cl (mmol/kg wet wt)	Mg	Ca	P	Dry wt g%	
Nucleus	Control	17.3 ± 14.2	110.6 ± 24.2	24.9 ± 12.8	8.7 ± 2.3	0.9 ± 0.9	130.8 ± 40.8	24.1 ± 5.2	(107)
	Ouabain	52.4 ± 43.0^{b}	68.8 ± 43.4^{b}	27.4 ± 11.1	8.0 ± 2.2	1.0 ± 1.2	114.1 ± 31.1^{b}	23.3 ± 6.5	(86)
	Amiloride	9.3 ± 6.9^{bc}	130.6 ± 36.2^{bc}	17.0 ± 12.4^{bc}	9.9 ± 2.6^{bc}	0.5 ± 0.7^{bc}	152.4 ± 72.3^{bc}	25.7 ± 8.5^{c}	(62)
	Am + Ouab	26.2 ± 31.7^{bcd}	103.1 ± 35.4^{cd}	24.7 ± 22.8^{d}	9.1 ± 2.6^{cd}	0.9 ± 1.1^{d}	125.7 ± 45.6^{cd}	25.6 ± 8.7^{c}	(112)
Cytoplasm	Control	21.6 ± 16.8	107.7 ± 22.8	34.5 ± 15.8^{a}	10.2 ± 2.9^{a}	1.2 ± 1.2^{a}	116.0 ± 36.4^{a}	28.0 ± 7.5	(101)
	Ouabain	56.0 ± 37.9^{b}	69.7 ± 43.5^{b}	34.7 ± 12.8^{a}	8.8 ± 2.9^{b}	1.0 ± 1.3	103.8 ± 36.2^{b}	27.6 ± 8.9	(72)
	Amiloride	10.9 ± 6.6^{bc}	126.0 ± 23.9^{bc}	25.5 ± 14.8^{abc}	11.4 ± 3.1^{ab}	0.9 ± 0.9^{a}	118.7 ± 52.7^{a}	30.2 ± 8.5^{bc}	(44)
	Am + Ouab	26.2 ± 29.7^{cd}	105.0 ± 34.8^{cd}	29.6 ± 16.8^{abc}	10.9 ± 3.4^{a}	1.3 ± 0.9^{ad}	133.4 ± 40.7^{bc}	31.9 ± 11.9^{bc}	(100)

Mean values ± SD of (*n*) individual measurements in nucleus or organelle-free cytoplasm. Superscripts denote the statistical significance at $2P < 0.05$ level: [a]different from nucleus; [b]different from control; [c]different from ouabain; [d]different from amiloride.

crease was most pronounced in those skins which experienced the largest inhibition of the I_{sc}. In contrast, the Na concentration was reduced after amiloride and after simultaneous application of amiloride and ouabain (Am + Ouab). After amiloride plus ouabain the Na concentration was slightly higher than after amiloride alone. All changes in the Na concentration were mirrored by equal but opposite changes in the K concentration, leaving the sum of the intracellular Na and K concentration almost unchanged.

In four out of seven experiments, application of ouabain resulted in a statistically significant reduction of the Cl concentration. Skins with a large Cl decrease generally also showed an increase in the dry weight content, suggesting some cellular shrinkage. The small increase in the Mg concentration may also be explained by cell shrinkage. In contrast, application of amiloride resulted in an increase of the Cl concentration. After amiloride, as well as after amiloride plus ouabain, a small but significant drop in the Ca concentration was detectable.

Besides nuclear values, Table 1 also lists the cytoplasmic concentrations of principal cells. In general, the Na, K, and Cl values in the cytoplasm closely follow the nuclear values; however, the Na and Cl concentrations are consistently higher than in the nucleus. Further systematic differences are detectable for Mg, Ca, and P and the dry weight content.

DIFFERENT TYPES OF MITOCHONDRIA-RICH CELLS

The large scatter in the Na concentration raises the possibility that the MR cell population is made up by different subpopulations of cells. To further investigate this question, we continued sectioning until in each experiment about 20 MR cells per condition were obtained.[1] Unfortunately, the tissue was often consumed before that number was reached.

The results were rather variable between skins. In some skins, the majority of MR cells showed a significant Na concentration increase after ouabain, in others ouabain had apparently no effect. Figure 5 shows an experiment in which the majority of MR cells (16 out of 24) experienced a large Na increase after ouabain. A much smaller proportion of cells (only 2 out of 16) showed high Na concentrations after amiloride plus ouabain, indicating that in most

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[1] Typically, a section contained only one MR cell. Average density of MR cells was 1.9/100 principal cells.

cells the Na influx is inhibitable by amiloride. Application of amiloride alone reduced the Na concentration compared to control (right panel of Fig. 5).

MR cells generally had a slightly lower Cl concentration than principal cells. However, with the exception of a few skins in which a distinct group of low Cl MR cells could be distinguished, the Cl values were considerably higher than those previously observed in MR cells of European frogs (Rick et al., 1978; Dörge et al., 1990). In the experiment illustrated in Fig. 5, most of the MR cells were of the high Cl type (>20 mmol/kg wet wt). While the Cl concentration was unchanged after ouabain, a significant reduction was detectable after amiloride. In skins with predominantly low Cl MR cells, amiloride had much less pronounced effects on Na and Cl.

MR cells with elevated intracellular Na often looked swollen. Figure 6 depicts the relationship between cell volume and Na concentration (same experiment as in Fig. 5). Clearly, in all cells with high intracellular Na the cell volume is increased. A positive, however much weaker, correlation between volume and intracellular Na is evident in control. After amiloride plus ouabain the number of swollen MR cells was significantly reduced (*data not shown*). Amiloride alone resulted in a small reduction of the cell volume compared to control (right panel of Fig. 6). Plotting the cell volume as a function of the intracellular Cl concentration revealed no significant correlation.

Figure 7 illustrates the relationship between the Na and Cl concentrations in MR cells based on the data from all seven individual experiments. Figures 8 and 9 depict frequency histograms for Na and Cl, respectively. Corresponding mean values are listed in Table 2.

Compared to control, slightly more than half of all MR cells shows a Na increase after ouabain (Figs. 7 and 8). In about half of these cells the Na increase could be prevented by amiloride. The existence of an amiloride-insensitive Na influx is best demonstrated by comparing amiloride alone with amiloride plus ouabain. Comparison of the two conditions also reveals that, in the presence of amiloride, ouabain increases the average Cl concentration, mainly by increasing the number of cells with low Na and high Cl. In the absence of amiloride, ouabain had no statistically significant effect on the Cl concentration, although the peak of the frequency distribution shifted somewhat to the right (Fig. 9). The Cl concentration of cells with high Na concentration (>30 mM) was not significantly different from those with low Na (25.7 ± 9.3 *vs*. 29.8 ± 12.9 mmol/kg wet wt $2P > 0.05$).

Application of amiloride resulted in a significant reduction of the Na concentration in MR cells. This is clearly evident from the disappearance of a small group of MR cells which, under control conditions, demonstrated elevated levels of Na and Cl (Fig. 7). The decline in the Na concentration remained statistically significant even after excluding these cells from analysis (from 13.1 ± 6.3 to 8.5 ± 5.2 mmol/kg wet wt, $2P < 0.001$). Amiloride also lead to a reduction in the Cl concentration. In the presence of ouabain, however, the reduction in the Cl concentration was only small and statistically not significant. Nevertheless, a marked change in the frequency distribution of intracellular Cl from a bell-shaped profile to a bimodal distribution can be observed (Fig. 9). A bimodal frequency distribution is also apparent after amiloride alone.

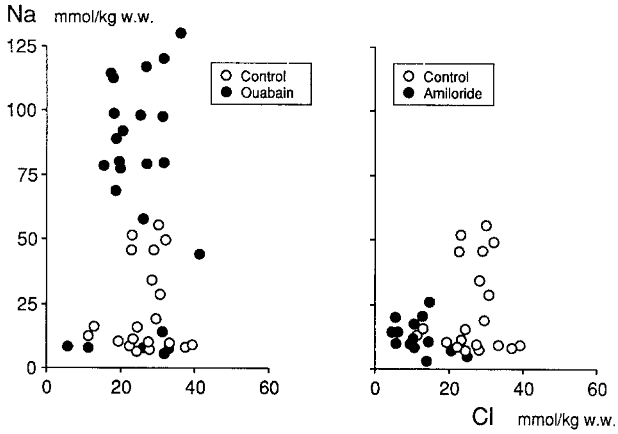

Fig. 5. Effect of ouabain (left) and amiloride (right) on the Na and Cl concentrations of MR cells. Data from 7–9 sections per experimental condition.

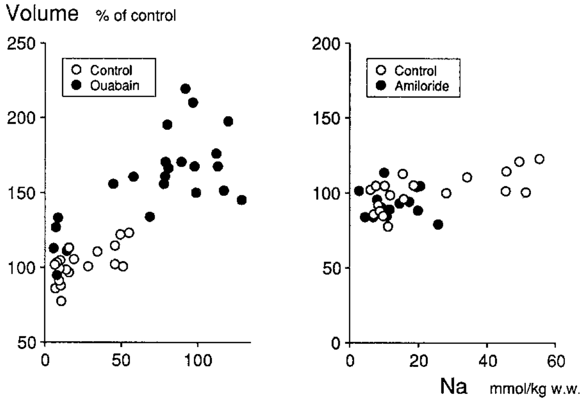

Fig. 6. Effect of ouabain (left) and amiloride (right) on Na concentrations and cell volume of MR cells. Same experiment as in Fig. 5. The cell volume was calculated from the dry weight measurement, assuming that changes in the dry weight content are caused solely by changes in volume. The average dry weight of MR cells in control (24.6 ± 4.7 g%) corresponds to 100% cell volume.

Variations in the Mg and P concentration and in the dry weight content of MR cells are consistent

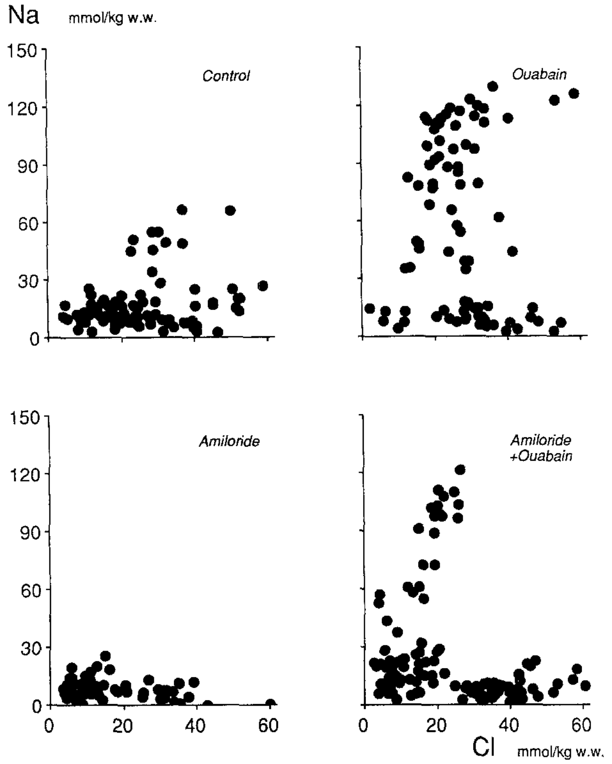

Fig. 7. Na concentration as a function of the Cl concentration in MR cells in control ($n = 107$), after ouabain ($n = 86$), after amiloride ($n = 62$), and after combined application of amiloride and ouabain ($n = 112$). Combined data from seven individual experiments.

with an average cell swelling of about 10% after ouabain and an about equivalent cell shrinkage after amiloride (Table 2). The values for amiloride plus ouabain are not significantly different from control, suggesting that there is no significant volume change under this condition. After amiloride, a reduction in the nuclear Ca concentration was detectable. Systematic differences between the nuclear and cytoplasmic concentrations were observed for Cl, Mg, Ca, P and the dry weight, however not for Na and K. In general, changes in the cytoplasmic concentrations paralleled those observed in the nucleus.

Discussion

Based on the intracellular Na concentration changes after amiloride and ouabain, at least three different types of MR cells can be distinguished: ouabain-insensitive cells, ouabain-sensitive and amiloride-insensitive cells, and cells that are sensitive to both ouabain and amiloride (Fig. 10). Roughly 50% of the MR cells were ouabain-sensitive, in about half of them the Na increase was inhibitable by amiloride. The data are inconclusive with regard to the question whether ouabain-insensitive cells may be further subdivided into amiloride-sensitive and amiloride-insensitive cells. Obviously, amiloride has an effect on the Na concentration even in the absence of Na/K pump inhibition (Figs. 5 and 7). None of the high Na, high Cl cells seen in control were detectable after inhibition of the apical Na influx by amiloride. It is unclear, however, whether these cells are ouabain sensitive or not.

In addition to lowering the Na concentration of MR cells, amiloride also lowered the Cl concentration. Based on the assumption that amiloride acts only on the apical Na channel, this result implies that the MR cell has a significant Cl conductance.[2] Inhibition of the Na influx by amiloride should lead to hyperpolarization of the membrane potential which, in turn, would stimulate electrodiffusive Cl efflux. Both after amiloride and after amiloride plus ouabain, a distinct population of cells with low Cl concentration could be detected (Fig. 9). Judging by the effect on intracellular Cl, at least half of all MR cells are sensitive to amiloride.

After ouabain, cells with high Na also experienced an increase in cell volume as judged by the cell size in cryosections and by the reduced dry weight content (Fig. 6 and Table 2). On the other hand, the average Cl concentration was not significantly changed. The lack of a Cl increase after ouabain is surprising since, based on the measured change in volume, swollen MR cells contained almost twice as many cations (Na + K) as normal cells. Unless the cells are capable of increasing the net negative charges of proteins or nucleic acids, it has to be assumed that swelling leads to the accumulation of another anion not visible in the analysis, such as bicarbonate.

According to our results, a distinct subpopulation of MR cells showed the behavior expected for a transepithelial Na transport compartment, i.e., a basolateral Na/K pump inhibitable by ouabain and an apical Na influx inhibitable by amiloride. The Na increase after ouabain was even more pronounced than in neighboring principal cells. This finding may be explained by a larger Na influx, as suggested by fast volume changes after amiloride (Larsen et al., 1987) or, alternatively, by a smaller pump activity, consistent with low ouabain binding (Mills et al., 1977) and low serosal uptake of Rb (Dörge et al.,

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[2] At the concentration used in this study, amiloride may also affect an apical Na/H exchanger, if present. In that case, the reduction in the Cl concentration could be explained by inhibition of a parallel Na/H and Cl/HCO_3 exchange system.

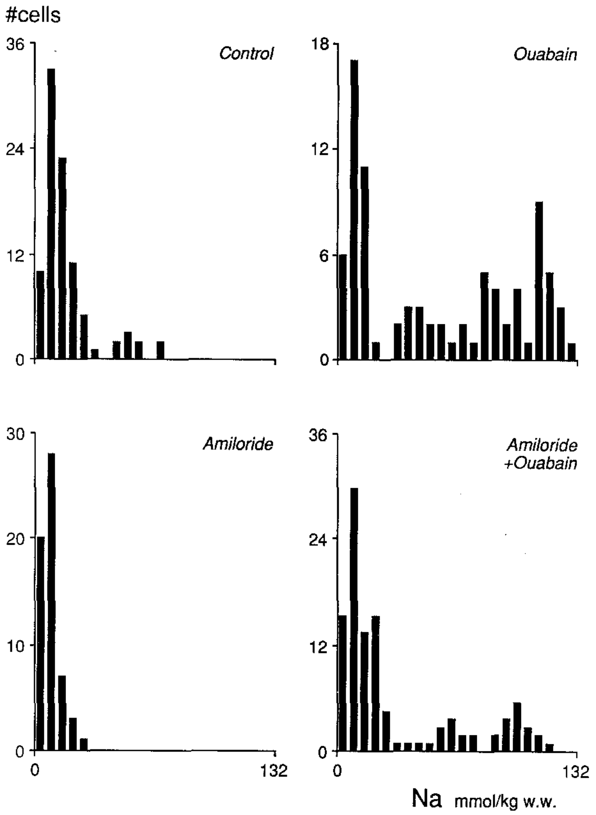

Fig. 8. Histogram of Na concentrations in MR cells. Same data as in Fig. 7. Bin width is 6 mmol/kg wet wt.

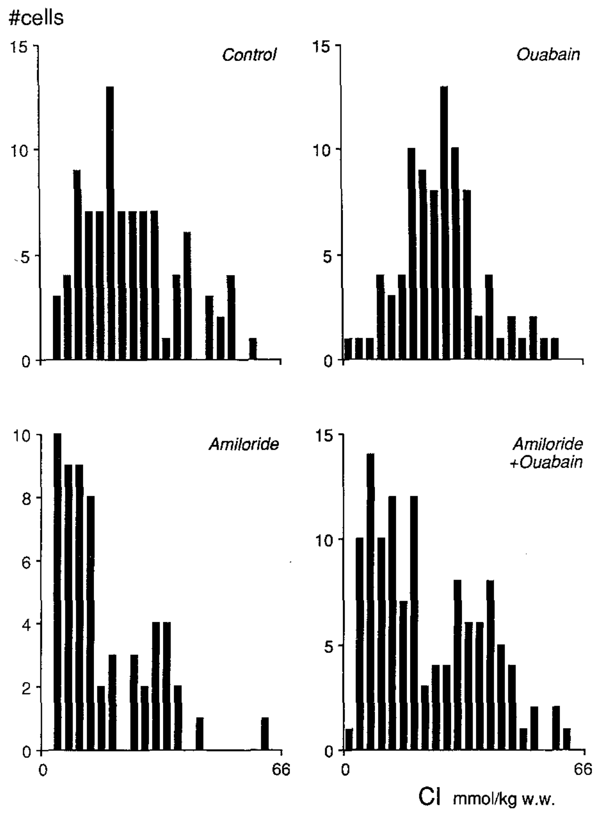

Fig. 9. Histogram of Cl concentrations in MR cells. Same data as in Fig. 7. Bin width is 3 mmol/kg wet wt.

1990). At any rate, because of the small number of cells, it is unlikely that this cell type contributes importantly to transepithelial Na transport under short-circuited conditions. The MR cell may, however, be of significance under in vivo conditions where the transport rate through principal cells is reduced by low external Na concentrations and by an inside-positive electrical potential difference.

In previous investigations using European frogs of the species *Rama temporaria* and *Rana esculenta*, no evidence for an amiloride-sensitive group of MR cells was obtained (Rick et al., 1978; Dörge et al., 1990). It should be noted, however, that the experiments were performed on cold-adapted frogs and intact skins rather than room temperature-adapted animals and split skins. In the present study a large number of MR cells with relatively high Na and Cl concentration was observed, whereas in both previous studies almost all the MR cells were of the low Na, low Cl type. Our data suggest that it is mainly the high Na, high Cl subtype which responds to amiloride.

A substantial number of MR cells demonstrated an amiloride-insensitive Na influx after ouabain. Since the bathing solution contained glucose, it is possible that the amiloride-insensitive Na influx takes place through the cotransport of Na and glucose. Of course, other Na-coupled pathways or the presence of an amiloride-insensitive Na channel cannot be ruled out. A further group of cells experienced an increase in the Cl concentration after ouabain, without any obvious change in the Na concentration (most clearly evident when comparing amiloride with amiloride plus ouabain in Fig. 7), suggesting that ouabain has effects on transport pathways other than the Na/K pump.

The group of ouabain-insensitive MR cells may be considered as the functional equivalent of MR cells in other epithelia, such as the intercalated cell of the mammalian collecting duct or the α and β cell of the turtle urinary bladder. Notably, all these cells are involved in the secretion and/or absorption of hydrogen ions and are assumed to be ouabain-insensitive (Steinmetz, 1986). Indeed, the amphibian skin is capable of secreting hydrogen ions (Huf, Parrish & Weatherford, 1951; Machen & Erlij, 1975), a transport function which has been recently localized to the MR cell (Larsen, 1991). The amiloride- and ouabain-sensitive group of MR cells probably corresponds to the γ subtype thought to be involved in transepithelial Cl transport (Larsen, 1991). A further cell population may correspond to precursor

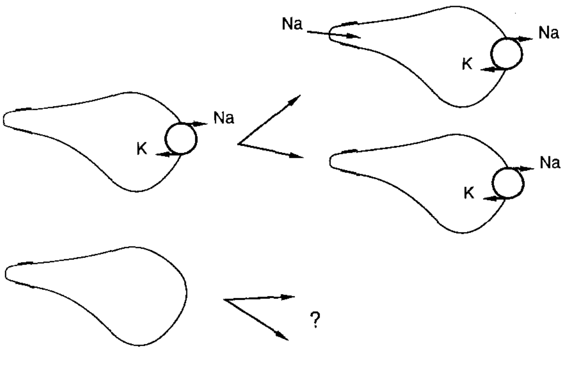

Fig. 10. Different types of MR cells in the frog skin epithelium.

cells that are not yet committed to a special ion transport function.

The frequency of ouabain-sensitive and insensitive cells and amiloride-sensitive and insensitive cells varied significantly from frog to frog, without any apparent reason. All frogs were maintained under identical conditions, obtained from the same supplier, and studied during the same time of the year (June/July). The variability may be explained by inter-individual differences or by nonrandom distribution (clustering) of different types of MR cells within the epithelium.

The heterogeneity of MR cells severely impedes the analysis of their transport function by electron microprobe analysis since the method compares the ion concentrations in different cells, rather than following changes in one and the same cell. This problem may be overcome if a particular type of MR cell can be selected for by adaptation of the frog to different salinities or different acid/base conditions. It has been shown that the isolated skin of *R. pipiens* is able to excrete either acid or base depending on the adaptation of the animal (Vanatta & Frazier, 1981). Metabolic acidosis results in an increased density of MR cells (Page & Frazier, 1987), providing evidence that the adaptation is achieved by the proliferation of a particular cell type rather than by transformation of cells.

With regard to principal cells, the results confirm our previous finding in *R. temporaria* and *R. esculenta* that the different layers of the epithelium form a syncytial Na transport compartment (Rick et al., 1978). The much smaller Na increase after ouabain can be explained by the lower concentration of ouabain used in the present study. Amiloride resulted in a significant decrease in the Na concentration in all epithelial layers and completely abolished the Na increase after ouabain, suggesting that virtually all the Na in principal cells originates from an influx through the apical Na channel.

Varying from the results in European frogs, a reduction in Cl concentration and in cell volume was detectable after ouabain. Both effects were abolished when the Na increase was prevented by amiloride. This result suggests that the Cl concentration in principal cells is determined by the combined Na and K gradient across the basolateral membrane which, in fact, will be more significantly affected by a small increase in the Na concentration than by the almost complete Na/K exchange that takes place after full inhibition of the Na/K pump. A Na-K-2Cl transport system in the basolateral membranes of principal cells has been previously demonstrated (Ferreira & Ferreira, 1981; Dörge et al., 1985).

The slight increase in the Cl concentration after amiloride may be explained by the increased Na gradient available for the Na-K-2Cl cotransporter. A comparable increase in the Cl concentration after amiloride was observed in vasopressin-stimulated skins (Rick et al., 1984). The finding of a rise in the Cl concentration conflicts with data obtained by ion-sensitive microelectrodes which demonstrated a large fall in cellular Cl activity following amiloride (Harvey & Kernan, 1984).

The increased Na gradient across the cell membrane may also account for the drop in the Ca concentration seen after amiloride and after amiloride plus ouabain. However, the putative Na/Ca exchanger in the basolateral membrane of principal cells cannot be the only system that maintains a low intracellular Ca concentration since, after ouabain, Ca was unchanged despite a fivefold increase in Na. Provided that the affinity of intracellular binding sites for Ca is unaffected by amiloride or ouabain, the observed changes in the concentration of Ca probably reflect changes in the intracellular Ca activity.

Occasionally, cells with very high Na and Cl concentrations were detectable in the basal cell layer, some of them apparently in the process of being sloughed off. The cells were grossly swollen and, as judged by their low Mg and high Ca content, had completely lost the integrity of the cell membrane. The loss of germinativum cells may explain why basal cells resembled spinosum cells (Fig. 1*A* and *C*). The damage to the basal cell layer is probably not only caused by the mechanical splitting of the skin, as swollen cells were detectable also in skins that were incubated with collagenase but kept intact (own unpublished results). The reduction in the total number of cells may account for the slight reduction in the I_{sc} compared to the intact skin.

The use of isolated epithelia significantly improved the freezing quality and allowed us to use much thinner sections for analysis. The increased spatial resolution power enabled us to make several observations. First, the lateral intercellular spaces are separated from the inner bathing medium by narrow exist slits (Fig. 1*C*). Albumin does not readily penetrate into the intercellular spaces, suggesting that the epithelial interstitium is not in totally free communication with the inner bath. The possibility has been raised that exit slits represent a significant barrier to transepithelial Na transport (Ziegler, 1976).

Second, in the deeper epithelial layers an occasional cell with very low Cl concentrations could be detected which otherwise had the normal appearance of a spinosum cell (Fig. 1*B*). The Cl concentration in these cells was much lower than in neighboring principal cells, making it unlikely that the cell is part of the syncytial Na transport compartment. The cell may represent a principal cell which has become uncoupled or a yet unknown epithelial cell type. Lymph cells and other white blood cells which also show low Cl concentrations can clearly be differentiated based on morphological criteria.

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