J. Membrane Biol. 151–165 (1978)

The Mitochondria-Rich Cell of Frog Skin as Hormone-Sensitive "Shunt-Path"

C.L. Voûte and W. Meier

The Laboratory of Experimental Nephrology, Department of Medicine–Kantonsspital, University of Basel, 4031 Basel, Switzerland

Received 30 November 1977

Summary. Further investigations about the role of the mitochondria-rich cell (MR cell) in hormone-mediated transport regulation in the epithelium of frog skin brought the following results: Unlike toad bladder, in frog skin the spontaneous potential difference cannot be reversed when Na transport is blocked. A similar situation is obtained when, in addition to transport-blockade, one applies a chemical gradient for chloride to the epithelium. Under these conditions we found that in the intact preparation as well as in the separated epithelium: (i) the reversed current (RC) is linearly related to the number of MR cells; (ii) RC is mainly carried by a passive, transcellular chloride flux inwards and (iii) RC is sensitive to nor-adrenaline (10^{-7} M). The beta-blocker propranolol abolishes this effect.

We propose that the MR cells are the sites of transepithelial shunt-path and that this chloride flux is transcellular. As it is hormone sensitive, it could be an important regulatory instrument for the regulation of overall salt transport (internal shorting).

The mitochondria rich or MR cells have been described in the epithelium of the frog skin ever since 1865 (Rudneff, 1865). That these cells could play a role as hormone receptors, however, was not recognized until a few years ago. Stimulated by results of Jørgensen (Jørgensen & Larsen, 1960) and Nielsen (1969) simultaneous morphofunctional survey brought evidence that these cells could be involved in aldosterone reception (Voûte *et al.*, 1969; Voûte, Hänni & Ammann, 1972) possibly by the intermediate role to synthetize and secrete a "permease" as postulated by, e.g., De Weer and Crabbé (1965). Subsequently, the last years brought a large amount of relevant information which may be summarized as follows:

In 1975 Zeiske and Lindemann reported that the permeability for sodium (P_{Na}) of the apical border membrane of frog skin epithelium could be increased by lowering the pH of the outer bathing fluid from 7.5 to 5.5. These results were in line with earlier observations of Leaf and collaborators (Leaf, Keller & Dempsey, 1964) that the acidification

0022-2631/78/S040-0151 $03.00

of the mucosal fluid in the toad bladder stimulates active sodium transport. Histochemically it has been shown that in the epithelium of frog skin carbonic anhydrase could be found exclusively in MR cells (Rosen & Friedley, 1973). Soon thereafter Scott and Sapirstein (1974) demonstrated that in enzymatically separated epithelial cells of toad bladder this enzyme could be found only in the MR cell fraction. In 1975 our group presented data favoring the idea that aldosterone induces carbonic anhydrase in MR cells only (Voûte, Thummel & Brenner, 1975*b*). In the kidney of adrenalectomized mice, Susuki and collaborators (Susuki, Ogawa & Inoue, 1974) were able to show that aldosterone stimulates only carbonic anhydrase, an effect which can be blocked by various protein synthesis inhibitors. Trying to find a common denominator for all these data, we tentatively formulated the hypothesis that the natriferic effect of aldosterone could indirectly be controlled by pH-dependent changes of P_{Na} across the apical border membrane of the granular cells (Voûte *et al.*, 1975*b*). In the same line a study by Ehrenfeld and Garcia Romeu (1976) brought proof for the secretory nature of these MR cells. Further important although indirect information in this question complex came from the Fanestil group in La Jolla. Ludens and Fanestil (1972) demonstrated that in urinary bladders of a certain species of toads the spontaneous potential could be reversed when active sodium transport is blocked, e.g., by amiloride. They showed that the reversed short-circuit current (RSCC) thus obtained is linearly related to the acidification of the mucosal bath. They also showed that this effect could be stimulated by aldosterone. The idea behind this approach forms, in a broad sense, the experimental base for the work we are reporting about in this paper.

It is well known that unlike toad bladder in frog skin the spontaneous potential does not reverse when sodium transport is blocked. All it does is to approach zero potential. At this point we have to describe the results of some earlier experiments. We had observed that in frog skin potential and short-circuit current could be reduced when a chemical gradient for chloride is applied to the skin by exchanging inside chloride by a nonpermeating anion like, e.g., gluconate. We had also observed that skins from winter animals behaved in this respect quite differently from skins of summer frogs or animals kept at room temperature. In winter skins one observes only a reduction of PD and SCC, whereas in summer frogs PD will reverse after application of the gradient even without blockade of sodium transport. It is well known that the main functional difference between the two kinds of skins, winter and summer, is found in the difference in their conductance for chloride and water

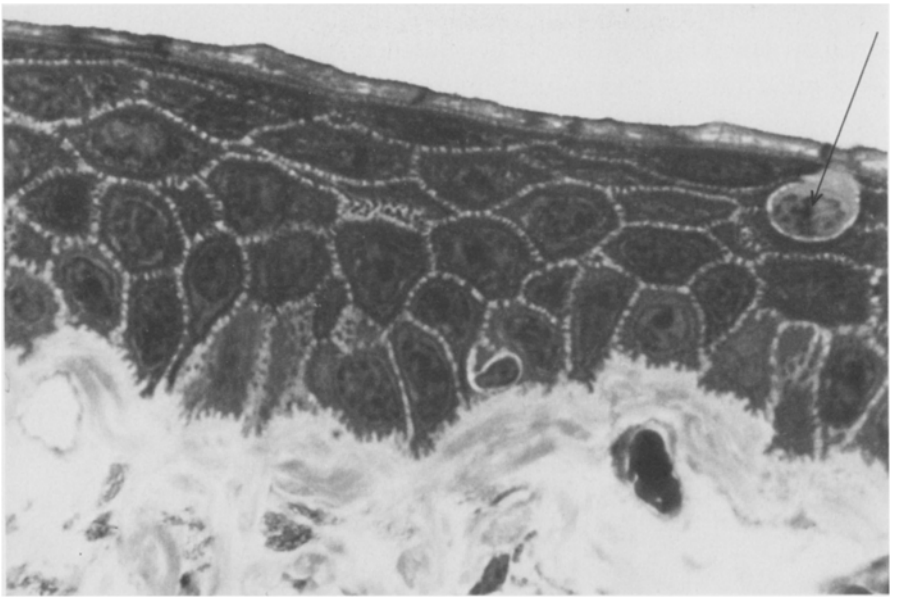

Fig. 1. Light micrograph of a frog skin epithelium from a winter frog. Low RC or Cl^- influx values. Arrow indicates an MR cell. Magnification 1,400×

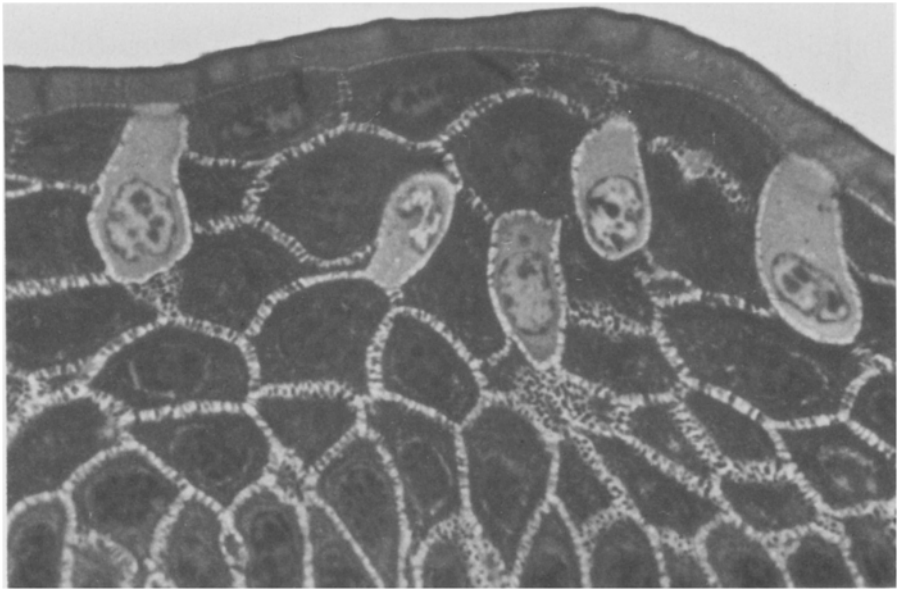

Fig. 2. Light micrograph of a frog skin epithelium from a spring frog. High RC or Cl influx values. Note the large number of MR cells, a few extending their neck to the subcorneal space. Magnification 1,400×

(Koefoed-Johnsen & Ussing, 1973). On the morphological side we had also observed a large seasonal dependence in the number of MR cells (*see* Figs. 1 and 2). Subsequent morphobioelectric experiments in this direction permitted us to demonstrate a linear relationship between RC (chloride conductance inwards) and the number of MR cells. In addition we could show that this chloride conductance reacts to nor-adrenaline in the full preparation and the separated epithelium.

Materials and Methods

With the exception of the isotope flux measurements (done by Dr. Kristensen in Copenhagen) on *Rana temporaria*, all animals used for this study were *Rana esculenta*. The frogs were kept in shallow tap water, either in a cold room or at room temperature, at least 3 days before used (in order to obtain skins with low and high chloride conductance). The experiments were done during the months December to May.

Abdominal skins were prepared the usual way and mounted in the experimental multi-chamber set as reported before (Voûte & Hänni, 1973). The composition of the various Ringer's solutions used can be found in Table 1. The preparation of the tissue pieces for morphological work was done according to the usual technique for light- and electron microscopy (e.g., Voûte, Møllgard & Ussing, 1975*a*). The procedure for obtaining separated epithelia is the one reported by Carasso, Favard, Jard & Rajerison, 1971.

Experimental procedure to Measure RC

a. Amiloride method (see Fig. 3). After an equilibration period of 1 hr in symmetrical NaCl Ringer's solution, the inside bathing fluid was replaced by Na-gluconate Ringer's. Active sodium transport was blocked subsequently by adding amiloride (10^{-4} M) to the outside solution. The majority of skins will now reverse their potential and after 15–20 min the reversed current (RC) is measured by short-circuiting PD.

b. K-Ringer's method. In this experimental series PD reversal is induced by blocking sodium transport through substitution of Na^+ by K^+ on both sides. An identical anion (Cl^-) gradient as in the amiloride method is created by replacing the inside solution by a K-gluconate Ringer's (in the presence of KCl-Ringer's outside).

Table 1. Ion concentrations in the Ringer's solutions used (mM)

	Chloride Ringer's	Potassium Ringer's	Na gluconate Ringer's	K gluconate Ringer's
Na^+	113.5	0	113.4	0
K^+	1.88	115.38	1.88	115.38
Ca^{2+}	1.08	1.08	1.08	1.08
Cl^-	115	115	0	0
HCO_3^-	2.4	2.4	2.4	2.4
Gluconate$^-$	0	0	115	115

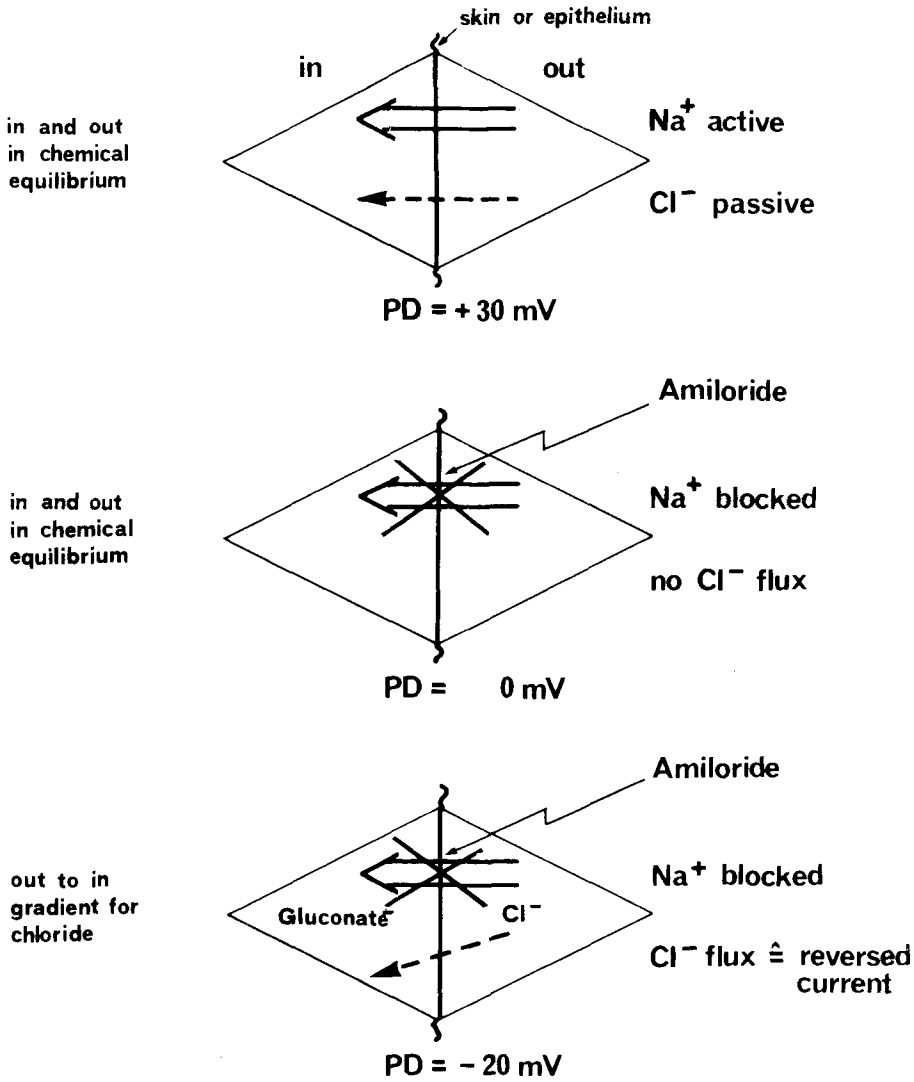

Fig. 3. Illustration of the procedure for obtaining PD reversal in frog skin (amiloride method). For details *see text*

In the hormone experiments (*see* Figs. 8 and 9) nor-adrenaline (10^{-7} M) is added to the inside solution 15–30 min after reversal of PD. In some experiments the beta-blocker propranolol was used inside one hour before adding the hormone (5.4×10^{-6} M).

Morphological Evaluation

From every experimental skin piece removed from the chamber three random squares of about 2×2 mm diameter were cut, fixed immediately and processed the usual way.

The blocks were cut in an ultramicrotome at a right angle to the skin surface. From every block three semithin sections (1 μm thickness) were cut, stained with Toluidine blue, and used for MR cell counts. The counts were done by two persons with a light microscope at a total magnification of 1200×, and every section was measured with a special device at a magnification of 40×. The cell counts were expressed in MR cells/mm section length.

Results

The first and critical step to do was the definition of the meaning and functional description of the term RC under the experimental condi-

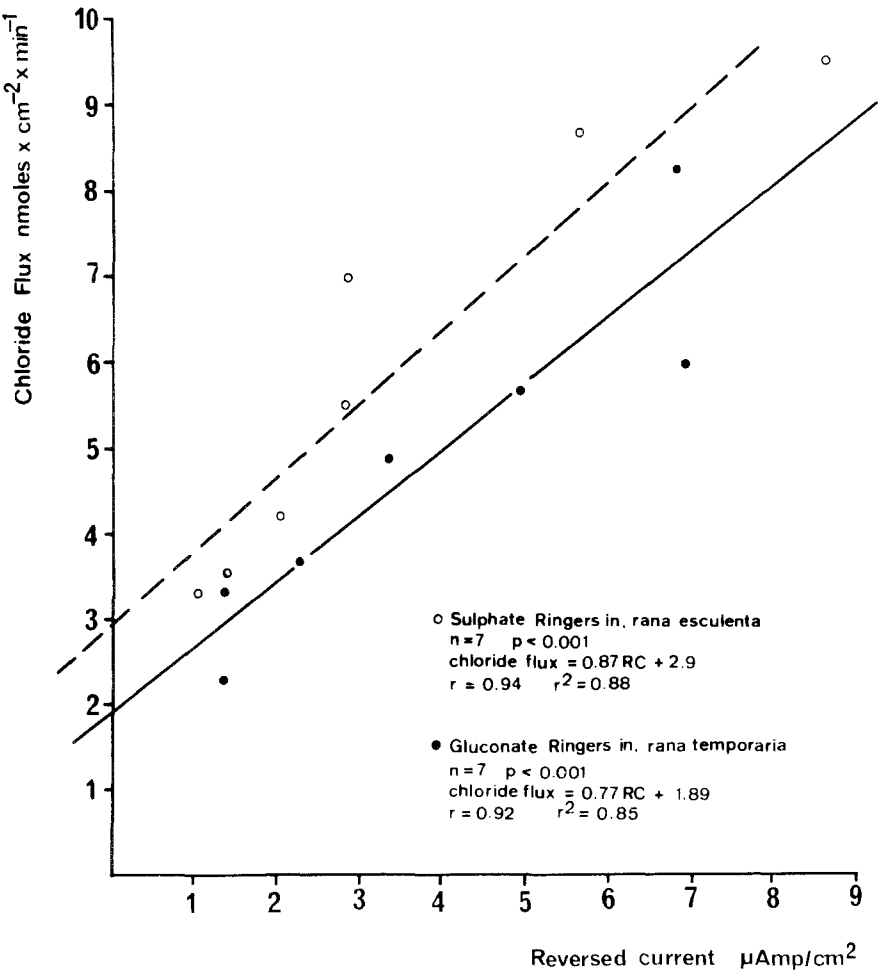

Fig. 4. Correlation between reversed current (RC) and chloride influx. Open circles stem from experiments with sulfate, full circles from those with gluconate inside

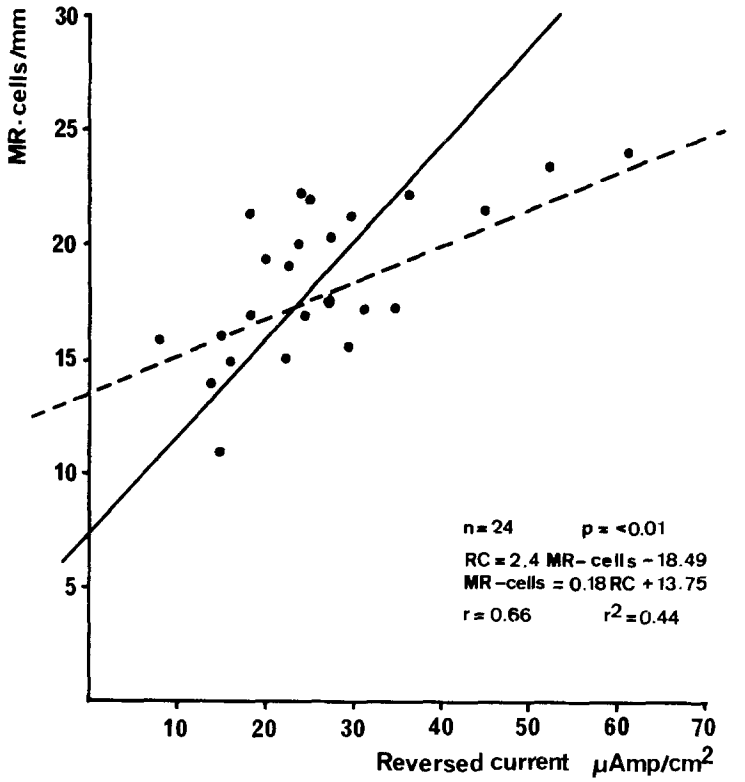

Fig. 5. Correlation between reversed current (RC) and number of MR cells (amiloride method). For discussion, *see text*

tions described and used in this series. The results of a first set of experiments stem from Dr. Kristensen (1977) and were done on skins of *R. temporaria* early in winter. Fig. 4 demonstrates the isotope estimation of chloride influx plotted *vs.* spontaneous RC under constant short-circuit conditions. One can see two sets of results: Open circles were done with the anion sulfate on the inside, whereas full circles correspond to our experimental approach, namely, gluconate Ringer's inside. From the linear correlation it comes out clearly that what we measure with RC in this experimental set up is, as expected, nothing else than the magnitude of passive chloride influx in the absence of active sodium transport or, in other words, the transport independent moiety of anionic shunt flux. Also—and the next paper of this Symposium (Kristensen, 1977) will be dealing with this point more in detail—it came out clearly from these experiments that the passive chloride influx was in most skins drastically reduced after blockade of sodium transport by amiloride.

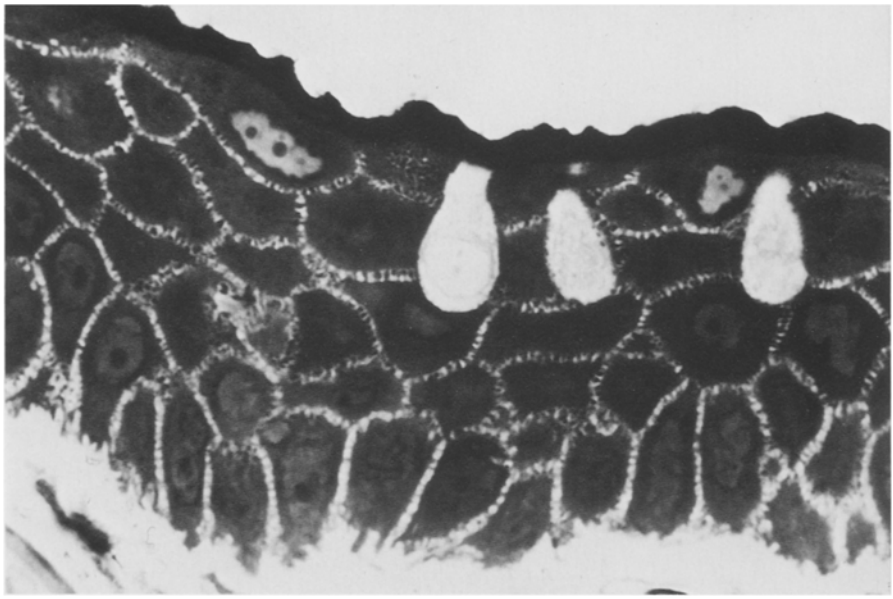

Fig. 6. Light micrograph of frog skin epithelium fixed under the experimental conditions of the K-Ringer's method (KCl-Ringer's outside, K-gluconate-Ringer's inside). Note the swollen MR cells as compared to the normal or shrunk granular cells. Magnification 1,400×

We will come back to this point when discussing the experiments in which sodium was replaced by potassium.

Fig. 5 shows the results we obtained from experiments in which we plotted RC values of the amiloride method against the corresponding MR cell counts. In spite of a large scatter of points, we found a significant correlation between the two parameters. Two points of insecurity and possible error source will be dealt with now. How efficiently does amiloride block sodium transport in frog skin, and how can we safely estimate the chloride conductance of every single MR cell counted? From SCC estimations and flux measurements in the literature, amiloride has a dose-dependent efficiency of about 90–98%. Thus, in the presence of a large transport-dependent chloride flux, even a small remaining part of this flux could give rise to a large error. Also, the counts could be misleading insofar that we simplify the functional state of the MR cells: We assume that they evenly contribute to chloride conductance, an assumption which is probably incorrect. However, as long as we cannot differentiate shunting from non-shunting MR cells on a morphological basis we had to look for another approach. Thus, as

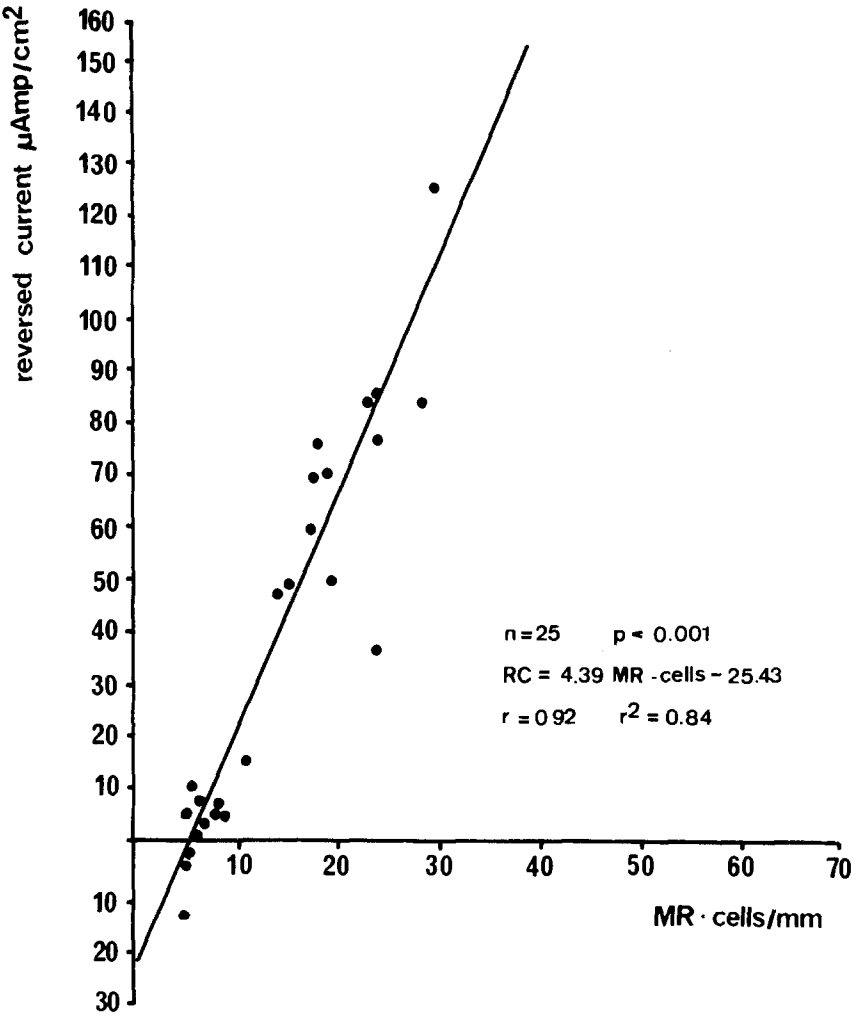

Fig. 7. Correlation between reversed current (RC) and number of MR cells (K-Ringer's method). For discussion, *see text*

a counter check another series of experiments was done in which sodium transport was not eliminated by a drug but by substituting sodium by potassium on both sides. By this new procedure the new factor of depolarization of either the transporting cell layer, the MR cells, or both would have to be considered. This situation should in the presence of a permeable anion call for a cell swelling (Ussing, Biber & Bricker, 1965). Under the experimental situation present in this series (gluconate inside), only cells permeable to outside chloride should swell (Koefoed-Johnsen *et al.*,

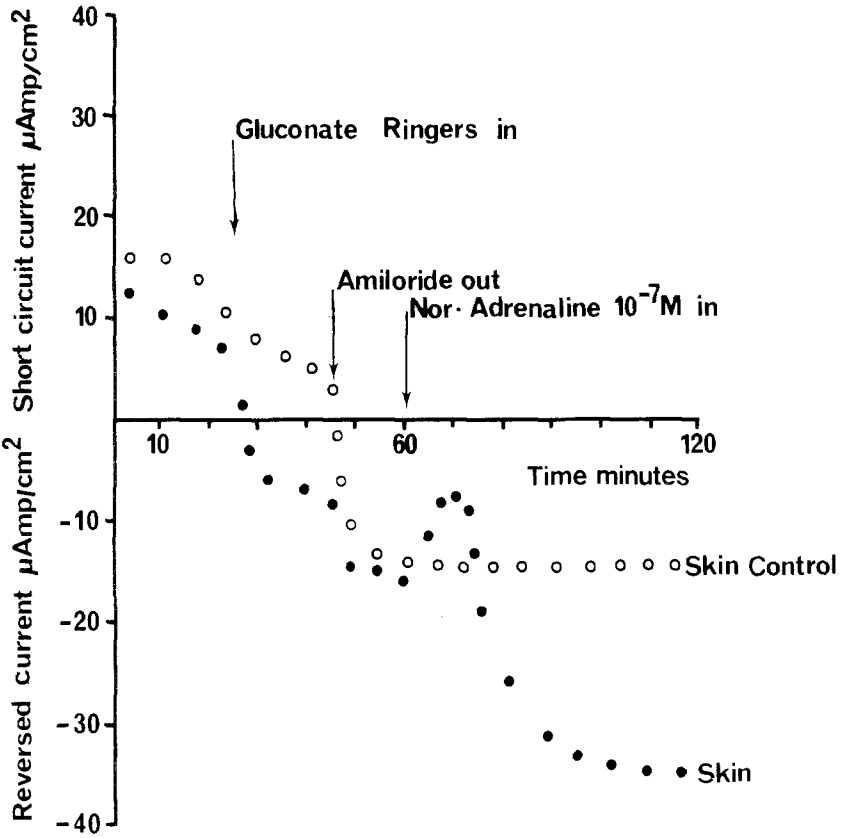

Fig. 8. Experimental protocol showing the effect of nor-adrenaline on RC of frog skin (amiloride method)

1973). Figure 6 illustrates the microscopic evaluation of this problem. One can see that the transporting cell layer looks either normal or even slightly shrunk (sometimes similar to the situation observed after adding amiloride). The only clearly swollen cells are the MR cells and apparently only if they have contact with the outside bathing solution. Figure 7 depicts the results of the MR cell counts plotted *vs.* RC under this new situation: K-Ringer's on both sides, chloride substituted by gluconate on the inside. A highly significant linear correlation is found between the two parameters, the higher the measured reversed current (passive chloride influx) the more MR cells are found. One should also note that the RC values measured with the K-Ringer's method are substantially higher than the ones with the amiloride method. We will come back to this point in the discussion.

Once the correlation between chloride influx and MR cells was estab-

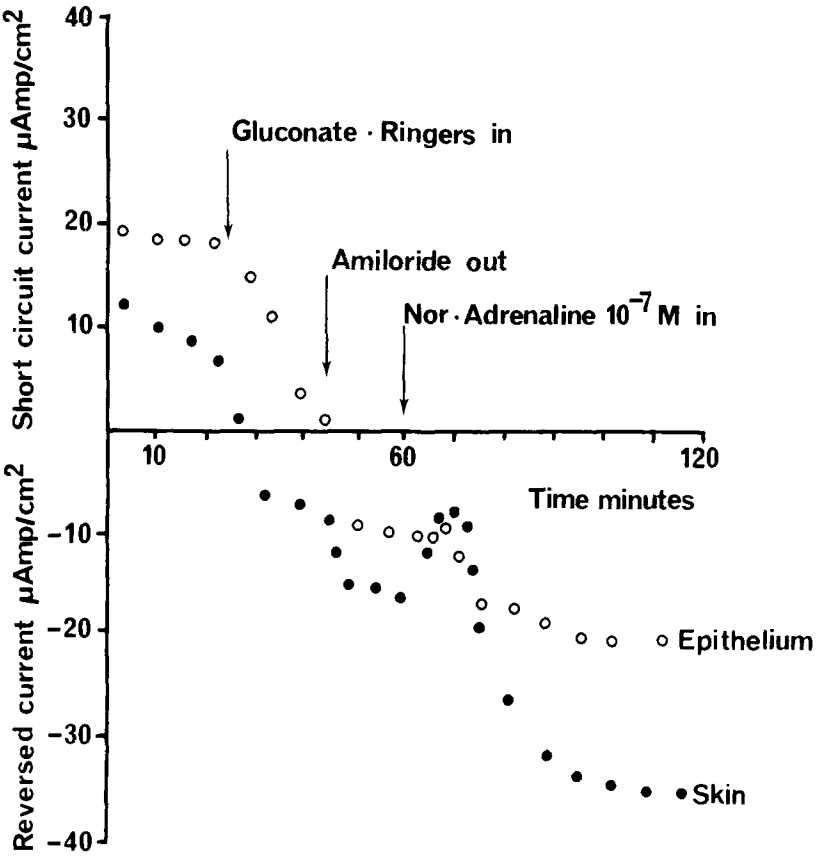

Fig. 9. Identical experiment as Fig. 8, however, depicting the effect of nor-adrenaline of frog skin as compared to the separated epithelium

lished, it seemed attractive to test the effect of hormones on the reversed current. The rationale behind this stems from the established facts that MR cells are secreting cells (glandular cells), that aldosterone can promote them to secrete carbonic anhydrase, and that in the epithelium of toad bladder the same hormone stimulates proton secretion in parallel to RSCC. Thus, it seemed logical to test, besides aldosterone, the action of hormones usually controlling glandular secretion, the ones of the adrenergic and cholinergic groups. The first hormone applied was nor-adrenaline. Figure 8 represents the protocol of a typical experiment. Two pieces of the same skin were prepared in two separate chambers. Both were submitted to the usual procedure for PD reversal. It should be mentioned that with both methods, the amiloride and the K-Ringer's method, one gets qualitatively the same response. In one chamber nor-

adrenaline (10^{-7} M) is added to the inside bathing solution. Whereas the control piece remains at a stable RC value, this parameter is drastically increased in the hormone-treated preparation. In order to check that this effect of the hormone has nothing to do with stimulation of the skin glands other than MR cells, we repeated the same procedure with pieces of separated epithelia as compared to whole skin. The protocol of a representative experiment is shown in Fig. 9. The response of RC to nor-adrenaline in the epithelium might be quantitatively reduced, however, it is clearly there and in the same direction.

All separated epithelia were checked morphologically and in no section could we ever find a single remaining skin gland. It should be mentioned that this described effect on RC can be fully blocked by the beta-blocker propranolol.

Discussion

On the basis of reported results we can formulate a few statements:

1 Under the experimental conditions described, the reversed current is carried by a passive inwards chloride flux across the epithelium of the frog skin.

2 This reversed current is linearly related to the number of MR cells.

3 This reversed current is sensitive to nor-adrenaline.

4 This reversed current is submitted to large seasonal variations.

Sticking strictly to these statements, we may be allowed to discuss some implications. As the results presented stem from an experimental preparation in the zero transport position, we are not going to discuss the effect of nor-adrenaline on the active sodium transport system. They are however, relevant in connection with the observations of House (1969) who found the hormone to increase chloride conductance of the frog skin epithelium. As already suggested by Garcia-Romeu and Ehrenfeld (1975) and Alvarado, Dietz and Mullen (1975), chloride influx is composed of two separate fractions, the sodium transport dependent, amiloride sensitive one, and the transport independent moiety which can be demonstrated only under disequilibrium conditions. Now we have to ask the question, "Why are we measuring two different values of chloride influx with RC under two conditions in which sodium transport is abolished: RC with Amiloride as transport blocker and RC in the presence of K-Ringer's as sodium substitution?" The main functional difference is found in the magnitude of chloride conductance under the two conditions: small with Amiloride, large with K-Ringer's. Confronting

critically these two situations, the crucial point will have to be looked for in the physiological effect exerted on the cells contributing potentially to passive chloride flux inwards, either the transporting granular cells (first living cell layer) or the MR cells, both being in contact with the outside bathing fluid. In the K-Ringer's experiments we find an obvious swelling of the MR cells with no change, or rather a shrinking, of the granular layer. The depolarizing condition in high K-Ringer's seems to increase chloride conductance through these MR cells only. This would then mean that even in highly permeable spring epithelia the apical membrane of the granular, transporting cells is virtually impermeable to chloride and that all chloride shunt goes through the MR cells. In the amiloride blocked preparation chloride conductance is much smaller, however still (admittedly with less good correlation) connected to the number of MR cells. This increased resistance to chloride flux with amiloride could be due to a hyperpolarization of the transporting cell layer (cell shrinking) in the absence of trespassing sodium. If on the other hand we accept the MR cells to be the site of shunt path, the difference would easily be understandable: With Amiloride a relatively high chloride resistance due to a normal cell polarity of the MR cells, a situation, however, drastically altered by the depolarizing condition in high K-Ringer's. Also, if all shunt would flow through the MR cells this would mean that also the transport dependent moiety would have to take that way. Thus, RC in K-Ringer's would somehow also be a measure (at least a limiting value) for the total shunt conductance, including the sodium transport dependent part. It ensues that a strict separation of transport dependent and independent chloride conductance becomes purely dogmatic and rather unrealistic.

If we recall some of the statements already made—the linear relation between RC and the number of MR cells; the swelling and shrinking phenomena under K-Ringer's; and the RC response to nor-adrenaline in the skin and separated epithelium—a satisfying explanation can only be found on the basis of a transcellular shunt path through the MR cells. It seems obvious that these cells can contribute to RC (or chloride conductance) only when in open contact with the chloride containing outside solution, a situation found in leaky spring and summer skins. Thus, it also seems clear that this situation can be forced by stimulating these cells to secrete (nor-adrenaline effect). This would also go along with the observation of Whitear (1974) that nerve endings in the epithelium proper are found in the vicinity of MR cells only. It could also be a clue as to why heavy metals seem in their early effect mostly

to change parameters of passive conductance (Koefoed-Johnsen *et al.*, 1973) and that, e.g., silver is precipitated almost selectively at the secretory pole of these same cells (Whitear, 1972).

An interesting question in this context is also: What role could these cells have as regulatory organs of seasonal transport adaptation in general and shunt conductance specifically? The potential importance of a hormone controllable shunt conductance in connection with the overall regulation of asymmetric salt transport by way of partial internal short circuiting has been mentioned before (Lindemann & Voûte, 1976). Finally, it is tempting to look for a correlation between RC described in this paper and the spontaneous RSCC of, e.g., toad bladder. RSCC is a true short-circuit current under equilibrium conditions and thus may be a good measure of active proton extrusion. In our preparation this active side seems to be absent unless there exists an electrically silent or strictly electroneutral underlying transport process; this, however, would not be measurable by RC nor would it contribute to this current. Thus, so far RC and RSCC have the only common denominator in that very likely they are connected to the functional role of the MR cells. And these cells in both tissues exhibit striking similarities as hormone receptors, as synthetising and secreting cells. If in addition they turn out to be the key to a hormone controllable shunt conductance, a new, fundamental, and potentially important role of these specialized cells might help to shed more light into parts of epithelial transport regulation.

The authors wish to express their gratitute to Mrs. Elfriede Chappuis and Mrs. Helene Fluri for their skillful technical assistance.

This work was supported by grant 3.052.73 of the Swiss National Science Foundation. Preliminary results have appeared as an abstract under the title: "Demonstration of a hormone sensitive 'shunt-path' in the frog skin epithelium". *Experientia* **33**:786 (1977).

References

Alvarado, R.H., Dietz, T.H., Mullen, T.L. 1975. Chloride transport across isolated skin of *R. pipiens*. *Am. J. Physiol.* **229**:869

Carasso, N., Favard, P., Jard, S., Rajerison, R. 1971. The isolated frog skin epithelium: I. Preparation and general structure in different physiological states. *J. Microsc.* **10**:315

De Weer, P., Crabbé, J. 1965. Le mode d'action de l'aldostérone. *J. Physiol. (Paris)* **57**:600

Ehrenfeld, J., Masoni, A., Garcia-Romeu, F. 1976. Mitochondria rich cells of frog skin in transport mechanisms: Morphological and kinetic studies of transepithelial excretion of methylene blue. *Am. J. Physiol.* **231**:120

Garcia-Romeu, F., Ehrenfeld, J. 1975. Chloride transport through the non-shortcircuited isolated skin of *R. esculenta*. *Am. J. Physiol.* **228**:845

House, C.R. 1969. The role of glandular activity in the electrical response of amphibian skin to noradrenaline. *J. Physiol. (Lond.)* **202**:631

Jørgensen, C.B., Larsen, L.O. 1960. Hormonal control of moulting in amphibians. Nature (London) **185**:244

Koefoed-Johnsen, V., Lyon, I., Ussing, H.H. 1973. Effect of Cu ion on permeability properties of isolated frog skin. Acta Physiol. Scand. **396 (Suppl.)** 102

Kristensen, P. 1977 Effect of drugs on chloride transport across amphibian epithelia. (*see* p. 167, this issue)

Leaf, A., Keller, A., Dempsey, F.E. 1964. Stimulation of sodium transport in toad bladder by acidification of mucosal medium. *Am. J. Physiol.* **207**:547

Lindemann, B., Voûte, C. 1976. Structure and function of the epidermis. *In:* Frog Neurobiology. p. 198. R. Llinas and W. Precht, editors. Springer, Berlin-Heidelberg-New York

Ludens, J.H., Fanestil, D.D. 1972. Acidification of urine by the isolated urinary bladder of the toad. *Am. J. Physiol.* **223**:1338

Nielsen, R. 1969. The effect of aldosterone *in vitro* on the active sodium transport and moulting of the frog skin. *Acta Physiol. Scand* **77**:85

Rosen, S., Friedley, N.J. 1973. Carbonic anhydrase activity in *rana pipiens* skin: Biochemical and histochemical analysis. *Histochemistry* **36**:1

Rudneff, M. 1865. Ueber die epidermoidale Schicht der Froschhaut. *Arch. Mikr. Anat.* **1**:295

Scott, W.N., Sapirstein, V. 1974. Partition of tissue functions in epithelia: Localization of enzymes in "mitochondria-rich" cells of toad urinary bladder. *Science* **184**:797

Susuki, S., Ogawa, E., Inoue, Y. 1974. Effects of aldosterone, actinomycin D, puromycin and cycloheximide on RNA synthesis, carbonic anhydrase and ATPase activities of the kidney and on urinary excretion of sodium in adrenalectomized mice. *J. Steroid Biochem.* **7**:429

Ussing, H.H., Biber, T.U.L., Bricker, N.S. 1965. Exposure of the isolated frog skin to high potassium concentrations at the internal surface: II. Changes in epithelial cell volume, resistance and response to antidiuretic hormone. *J. Gen. Physiol.* **48**:425

Voûte, C.L., Dirix, R., Nielsen, R., Ussing, H.H. 1969. The effect of aldosterone on the isolated frog skin epithelium (*R. temporaria*): A morphological study. *Exp. Cell Res.* **57**:448

Voûte, C.L., Hänni, S. 1973. Relation between structure and function in frog skin. *In:* Transport mechanisms in Epithelia. H.H. Ussing and N.A. Thorn, editors. p. 86. Munksgaard, Copenhagen

Voûte, C.L., Hänni, S., Ammann, E. 1972. Aldosterone induced morphological changes in amphibian epithelia *in vivo*. *J. Steroid Biochem.* **3**:161

Voûte, C.L., Møllgard, K., Ussing, H.H. 1975*a*. Quantitative relationship between active sodium transport, expansion of endoplasmic reticulum and specialized vacuoles ("scalloped sacs") in the outermost living cell layer of the frog skin epithelium (*Rana temporaria*). *J. Membrane Biol.* **21**:273

Voûte, C.L., Thummel, J., Brenner, M. 1975*b*. Aldosterone effect in the epithelium of frog skin: A new story about an old enzyme. *J. Steroid Biochem.* **6**:1175

Whitear, M. 1972. The location of silver in frog epidermis after treatment with Ranvier's method, and possible implication of the flask cells in transport. *Z. Zellforsch.* **133**:455

Whitear, M. 1974. The nerves in frog skin. *J. Zool.* **172**:503

Zeiske, W., Lindemann, B. 1975. Blockage of Na-channels in frog skin by titration with protons and by chemical modification of COO^- groups. *Pfluegers Arch.* **355**:R71