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Volume Changes and Potential Artifacts of Epithelial Cells of Frog Skin following Impalement with Microelectrodes Filled with 3 M KCl

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Summary. Cells of isolated frog skin epithelium were observed microscopically during impalement with standard microelectrodes of 5 to 20 MΩ resistance, filled with 3 M KCl. Impaled cells, as well as some neighboring cells, were seen to swell 10 to 100 sec after impalement, while the negative potential recorded by the microelectrode depolarized (open circuit conditions). Apparently, osmotic swelling of small epithelial cells may be caused by diffusion of KCl from such electrodes. This conclusion is supported by calculations quoted from the literature of KCl loss from microelectrodes.

Intracellular recordings from epithelia with destructed cellular membranes gave negative "pre-tip potentials" of up to 18 mV. The potentials could be altered by electrode movement, by decreasing the ambient pH or the tip-pH and by modifying the fixed charges of the tissue chemically. It is shown that even a moderate loss of KCl, which will not result in appreciable swelling, can produce negative potentials in front of the electrode tip if the protoplasm has a high density of negative fixed charges.

We suggest the use of 3 M KCl electrodes with resistances above 30 MΩ if after impalement compression of intracellular material by the tip can be avoided. Where such compression cannot be avoided, it is best to fill the microelectrode with an isotonic solution which mimics the electrolyte composition of the cytosol.

Intracellular potentials have often been recorded from amphibian skin epithelium (Table 1). The results reported differed considerably and, therefore, necessitate a detailed reinvestigation of possible methodological problems. In the previous studies, with one exception [35], the probing microelectrode was advanced from the outer solution so that it had to pass the *Stratum corneum* before impaling the first living cellular layer (outer *Str. granulosum*). As the corneal cells, and to a lesser extent the cellular layers beneath, are mechanically rigid and offer considerable resistance to electrode advancement, relatively sturdy electrode tips (large diameter, low resistance) were generally used. In most studies the tips

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Table 1. Characteristics of electrodes which were used in electrophysiological studies of frog skin or toad skin epithelia[a]

Authors	Reference	Filling M KCl	MΩ	Tip ϕ μm	Optical magnif. ×
Biber *et al.*	2	3	1– 5	0.5	–
Biber & Curran	3–4	3	13–22		–
Cereijido & Curran	6	3	1– 5	0.5	–
Chowdhury & Snell	7–8	2	10–30	0.3	400
Engbaek & Hoshiko	12	3	4–23		–
Helman & Fisher	16	3	20–40		–
Hviid Larsen	17	2.5	5–15	0.2	–
Lindemann & Thorns	24	3	5–50		400
Martin & Curran	25	3	1– 3	0.5	–
Nagel	26–31	3	5– 9	0.5	100
Nunes & Lacaz Vieira	33	3	2–10		–
Ottoson *et al.*	34	?	?	0.5–1	?
Rawlins *et al.*	35	3	5–30		100
Scheer & Mumbach	37	3	3–20	10	–
Schmidt	38	3			–
Ussing & Windhager	39	3	2–20		100
Whittembury	40	3	3–30		100

[a] Where binocular dissection microscopes were employed, an optical magnification of 100 × is assumed.

were filled with 3 M KCl. It may be suspected, therefore, that in these experiments the electrode tips lost KCl in amounts significant with respect to the osmotic content of small epithelial cells. A change of cell volume was not observed, however, since these experiments were, with few exceptions [7, 8, 24], done either without optical control or under low power binocular dissection microscopes, which do not permit a reliable observation of the impaled cell.

To clarify this methodological problem, we impaled frog skin epithelia from the outside, using the electrodes typical for most of the previous frog skin studies, while observing the impalement process under 400- to 500-fold magnification. In order to achieve the improved optical conditions, two concessions had to be made in which our experiments differed from most of those listed in Table 1: (i) We employed isolated epithelia in order to be able to use transillumination, and (ii) we advanced the electrode, not vertically to the epithelial surface, but at a shallow angle of about 30°. We, thereby, gained the dual advantage of being able to see the recording approximate position of the tip and of being able to

distinguish from which cell type and at which distance from the epithelial surface the potential was recorded.

Many impaled cells in the *Str. granulosum*, including mitochondria-rich cells, swelled spontaneously in the first 10–100 sec after impalement when 3-M KCl electrodes of resistances up to 20 MΩ were used. The swelling or a characteristic change in optical appearance was often shared by one or several neighboring cells. Swelling could be avoided by using electrodes filled with solutions approximately isotonic to the cellular interior. Therefore, as may be expected, the use of low resistance electrodes filled with hypertonic solutions appears to introduce considerable osmotic artifacts.

The injection of KCl into impaled cells may also be expected to cause changes of membrane properties which result partly from the volume change and partly from the increase in cellular K and Cl concentrations. Indeed, the electrical properties of impaled cells changed during swelling. They were, thus, different from those of unimpaled cells and could not be relied upon as being typical for the tissue.

In order to find out whether the potentials recorded prior to visible swelling are reliable, we used epithelia with destructed cell membranes. Negative cellular potentials of up to 18 mV were then recorded with the 3-M KCl electrodes. These "pre-tip potentials" were smaller when electrodes of higher resistance were used but became larger again when such electrodes were advanced, compressing protoplasmic material in front of the tip. The potentials became positive after a chemical treatment of the tissue proteins, which removes negative fixed charges. After subsequent removal of positive fixed charges, the potentials decreased to low values. In contrast, isotonic electrodes recorded approximately zero potentials under all conditions. We conclude that 3-M KCl electrodes can record potentials from protoplasm which depend on sign and density of protoplasmic fixed charges. In intact cells these potentials are presumably additive to the membrane potentials.

Our results strongly suggest that microelectrodes filled with hypertonic KCl (i) cause osmotic artifacts in small cells and (ii) cause electrical artifacts when the cellular interior contains a high density of fixed charges. The use of such microelectrodes is less critical when the electrode resistance exceeds 30 MΩ and compression of protoplasmic constituents can be avoided while recording. Where compression cannot be avoided, electrodes filled with suitable isotonic saline are recommended.

Preliminary results were presented at the 1976 Fall Meeting of the "Deutsche Physiologische Gesellschaft" [11].

Materials and Methods

Isolated abdominal skin of *Rana esculenta* was used. Animals were kept at 12–15 °C with access to running tap water. Most experiments were done in April and May. The epithelium was separated from the corium by a collagenase method [5] and mounted outside up in a Lucite chamber suitable for microscopic observation. The chamber, a slightly modified version of one described previously [24], left 1.7 cm^2 of epithelium exposed to the bathing solutions. The inside bathing solution was Na-sulfate Ringer's containing, in some of the experiments, Eagle tissue culture medium; the outside bathing solution was Na- or K-sulfate Ringer's. The outside solution could be changed by a combination of gravity-feed and water suction.

Microelectrodes were drawn on a vertical puller from Schott glass tubing (outer diameter 1.2 mm, inner diameter 0.75 mm). They were filled *in vacuo* with methanol (100%) for 20 min, immersed in distilled water for 4 hr, then placed into either 3 M, 0.5 M or 0.1 M KCl, K-sulfate Ringer's, or 160 mM K-citrate plus 40 mM KCl overnight and used on the next day. In most experiments, however, borosilicate glass tubing (Hilgenberg Glass, Malsfeld, W. Germany) (outer diameter 1.25 mm, inner diameter 0.72 mm) with an internal glass filament was used, whereby the electrode could be filled with the desired solution immediately after pulling (Figs. 3, 4*B*, 5, 6, 7*D*, 8, 9, 10). Results obtained were independent of electrode glass or filling procedure. Electrodes filled with 3 M KCl, of resistances between 4 and 30 MΩ, were generally used. 3 M KCl electrodes of 16–20 MΩ (tip in Ringer's) had inner tip diameters of about 0.2 μm, as measured with a scanning electron microscope. Microelectrodes were mounted on a Leitz manipulator and advanced through the outer bathing solution at an angle of 30° to the horizontal epithelium. The microscope was fitted with a 40× water immersion objective (Zeiss) and a camera. Transillumination was employed.

The experiments were done in the current clamp mode, i.e., open circuit except for inward going rectangular current pulses of 50 msec duration and 10–50 μA/cm^2 density. Each current pulse was preceeded by an 8-msec, 0.1–1 nA pulse passed through the microelectrode to record the "input resistance." The following 5 signals were recorded continuously on paper-chart after passing through a demodulating sample-hold processor circuit[1]. V: Transepithelial voltage, outside *vs.* grounded inside bathing solution; V_e: Microelectrode potential *vs.* potential of inside solution; R, R_e: Resistive voltage deflection in response to the transepithelial current pulse, recorded transepithelially (R) and between microelectrode and inside solution (R_e); R_{ip}: Input resistance seen by the microelectrode amplifier. The shape of the recorded pulses was always checked by viewing the preamplifier outputs on an oscilloscope.

With the microelectrode tip in the outer solution, tip potentials of a few mV were usually observed. 3-M KCl electrodes (internal fiber type) dipping into Ringer's had mean tip potentials of −1.2 mV when R_{ip} was 8 MΩ, and −2.4 mV when R_{ip} was 16 MΩ, measured by breaking the tip. These potentials, which made V_e deviate from V, were electronically subtracted. The V_e values reported are potentials left after this correction. In order to investigate changes of microelectrode potentials, which occur when the microelectrode is placed into a cell, membrane potentials were eliminated by destructing the membranes mechanically through the formation of ice crystals. For this purpose the microscope chamber containing the epithelium was simply transferred into a deep freeze-refrigerator (−20° C) for 10 min, then returned to room temperature. The freeze-thawing reduced R and V to very small values. Subsequent impalement of cells was performed in the

1 Ganster, G., Lindemann, B. 1978. A processor for continuous recording of voltage and resistance from epithelial cells. (*in preparation*)

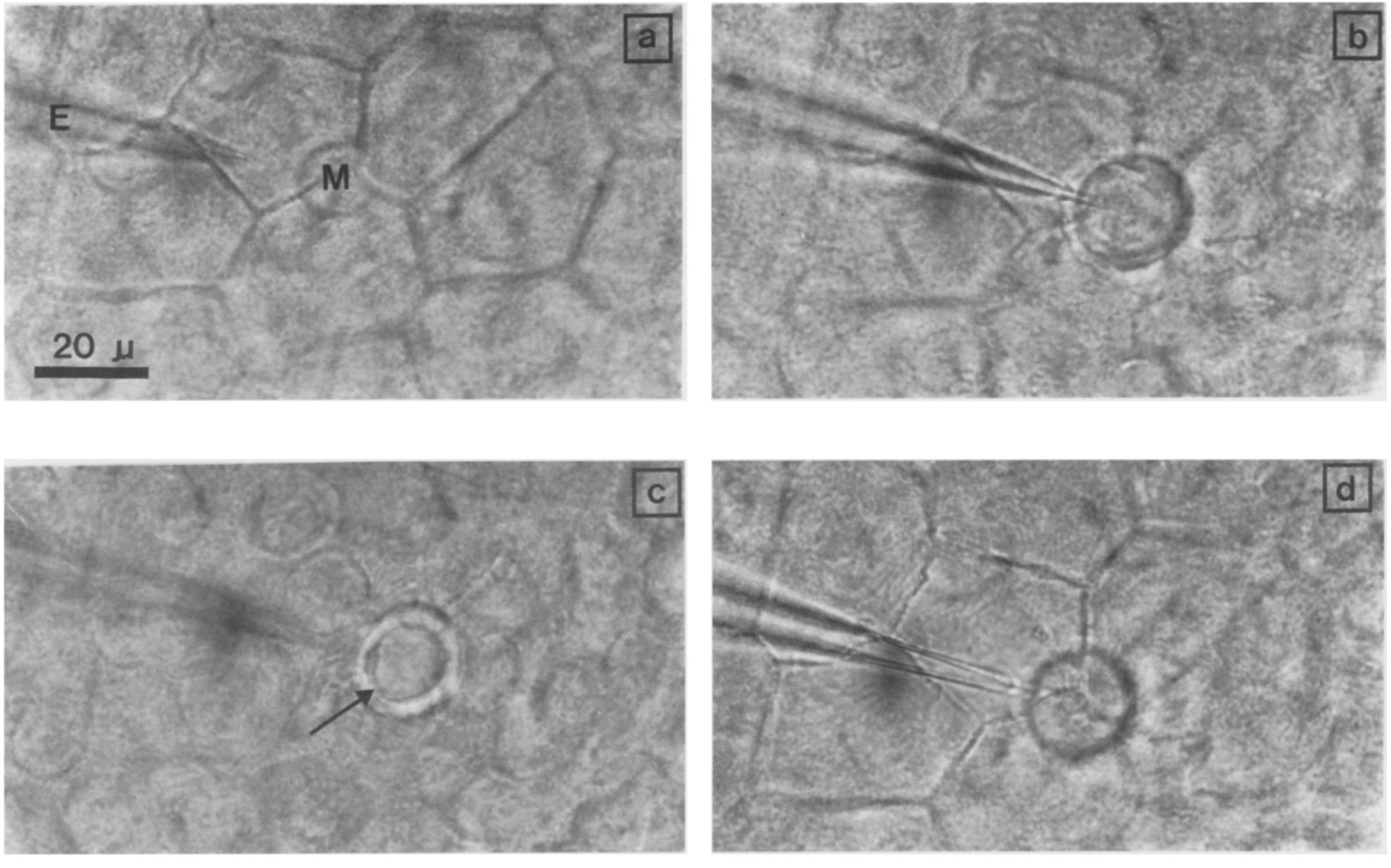
usual way. In such cases, K-sulfate Ringer's was used as the bulk medium in order to keep a high K concentration within the cytosol.

Results

Optical Observations

The microelectrode was positioned on the outer surface of the *Str. corneum* with its tip pointing, for instance, at a mitochondria-rich cell (MRC) selected for puncturing. The MRCs could clearly be identified in the *Str. granulosum* by their round cross-sectional appearance (Fig. 1*a*). After taking a control photograph, the microelectrode was advanced through the *Str. corneum* into the MRC. This caused some deformation of the tissue, mainly in the horizontal, and to a lesser extent in the vertical plane. The extent of deformation varied between preparations. Those epithelia where deformation was large could not be used. In favorable cases, the tip could be seen to indent the surface of the MRC at the level of the outer *Str. granulosum* and finally penetrate it. Due to the shallow impalement angle the position of the electrode tip could usually be estimated by focusing downward along the electrode shaft to the point where the electrode image vanished. The distance between this point and the surface of the epithelium was obtained by means of the focus calibration of the microscope.

On impalement with a microelectrode containing 3 M KCl, the MRC was in most cases observed to swell either immediately or with a delay of some 10–100 sec. In many cases the lateral cellular diameter was doubled. The cellular nucleus also increased in diameter (Fig. 1). The swelling was in most cases accompanied by an immediate or delayed change in optical appearance of one or more neighboring granular cells. These cells now seemed coarsely "granulated" and often also increased in lateral diameter (Fig. 2). MRCs bordering these granular cells were sometimes included in the swelling process. Often the extracellular space between and around the swollen cells became visible as a bright line of up to 2 μm in diameter, and in some cases the swollen MRCs developed one or several blunt surface protrusions of light appearance (Fig. 2). Similar "blebs" have been observed by Giulian and Diacumakos after pressure injection of Thorotrast into Hela cells [15]. While the swollen state persisted in most cases, sometimes the impaled and swollen MRC was seen to collapse 30–200 sec after impalement. Direct impalement of


a
E
M
20 μ
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d

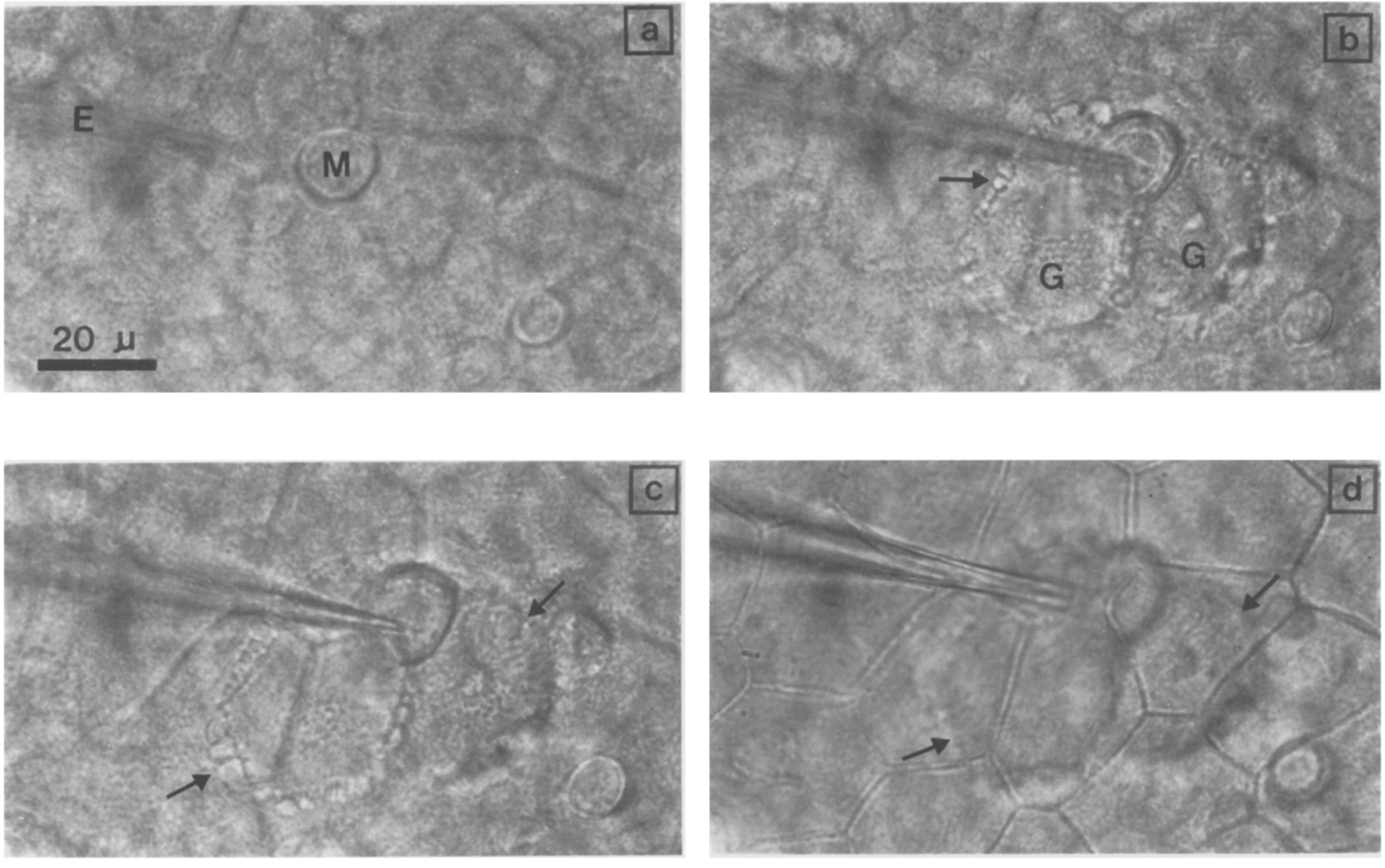
granular cells caused the same optical changes, which often involved neighboring MRCs.

Swelling was almost regularly seen with microelectrodes filled with 3 M KCl, having a resistance of 4–20 MΩ, less regularly with electrodes containing 0.5 M KCl. Swelling could not be avoided by filling the microelectrode shank with 3 M KCl-agar rather than with 3 M liquid KCl. Furthermore, it could not be prevented by replacing the outside Na by K or by adding amiloride to the outside solution. Swelling was not clearly affected by passage of current through the microelectrode. Swelling did not occur when microelectrodes filled with 0.1 M KCl, with K-sulfate Ringer's, or with a mixture of 140 mM K^+-citrate plus 30 mM KCl were used.

Electrical Changes during Impalement with 3-M KCl Electrodes

Impalements were usually done with Na-sulfate Ringer's on the outer surface of the epithelium. When the electrode tip passed through the *Str. corneum* (Fig. 3, label *i*), large, irregular positive and negative potentials were recorded while the tissue was visibly deformed and the input resistance R_{ip} rose to large values as described previously [24]. After subsequent impalement of the selected MRC or granular cell, the electrode was slightly withdrawn (label *j*) to effect an at least partial release of tissue compression. Thereby, R_{ip} decreased and V_e settled to a value which, in this case, was negative by 44 mV with respect to the outer solution (the mean value of more than 60 punctures with 7 MΩ electrodes was −21 mV, see Table 2, time t_1). This negative potential, apparently measured across the apical membrane, was accompanied by a low value of the R_e signal, indicating that a resistive barrier had been passed.

Fig. 1. Photomicrographs (*a*) through (*d*) illustrate the phenomenon of cellular swelling which was observed following impalement of frog skin epithelial cells with 3-M KCl microelectrodes. During this impalement there was no electrical connection between microelectrode and amplifier, thereby eliminating the possibility that the swelling phenomenon could result from current injected into the cell in the continuous monitoring of the electrode resistance. (*a*): Mitochondria-rich cell (*M*) prior to impalement; note the microelectrode (*E*) which appears out of focus in the upper lefthand corner. (*b*): Mitochondria-rich cell following impalement; the cellular diameter is increased by a factor of 1.7. The cell is surrounded by a white halo which is probably extracellular space. (*c*): Same mitochondria-rich cell viewed in a deeper plane of focus. Note the now sharp delineation between extracellular space and surrounding tissue. Arrow points to presumable border of swollen cell. (*d*): Here, the "chicken-fence" pattern of the *Str. Corneum* is in the plane of focus. The swollen mitochondria-rich cell can be seen beneath, outlined by a dark ring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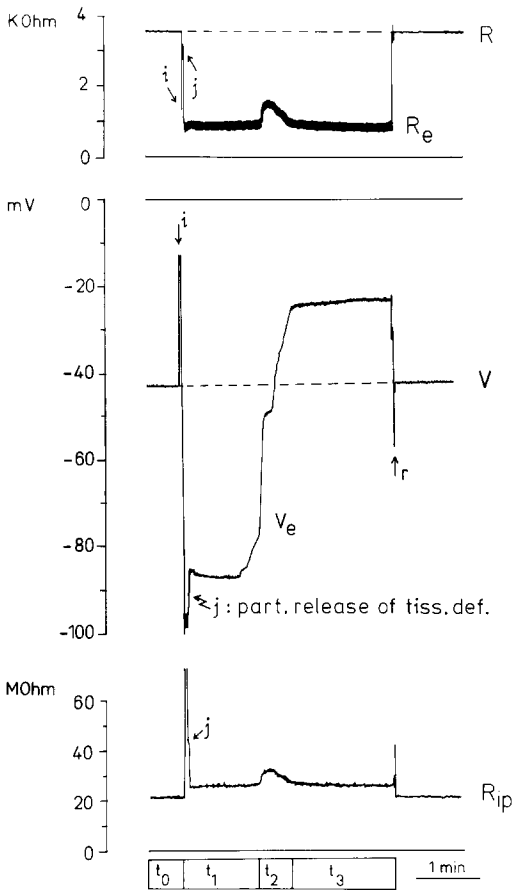

Fig. 3. Changes in electrical parameters associated with swelling of frog skin epithelial cells following impalement with a 3-M KCl electrode. The curves were obtained simultaneously after impalement of a granular cell of the outer *Str. granulosum* with an electrode of 21 MΩ initial resistance. The epithelium was not externally short-circuited. After impalement (label i) the electrode was slightly withdrawn (label *j*) to effect a partial release of tissue deformation. The tip position then remained unchanged until final withdrawal from the cell (label *r*). *R*: Transepithelial resistance of 1.7 cm^2 area; R_e: Resistive voltage deflection recorded through the microelectrode in response to the transepithelial current pulse; *V*: Transepithelial voltage, outside *vs.* grounded inside bathing solution; V_e: Microelectrode potential *vs.* potential of the inside solution; R_{ip}: Input resistance seen by the microelectrode amplifier; t_0: Time prior to impalement; t_1: Time directly after impalement; t_2: Time of rapid swelling and of transient electrical responses; t_3: Time of constant readings following rapid swelling. Outside and inside bathing solution Na-sulfate Ringer's

Fig. 2. Joint cellular swelling following impalement of a mitochondria-rich cell with a 3-M KCl electrode. (*a*): Mitochondria-rich cell (*M*) prior to impalement; note the microelectrode (*E*). (*b*): Mitochondria-rich cell after impalement; note the two adjacent granular cells (*G*) which have also changed optical appearance. Their protoplasm appears coarsely granulated (or vacuolated?), and their cellular borders are marked by the presence of what appear to be numerous protruding vesicles (blebs) lying, presumably, in the extracellular space surrounding the cells (arrow). (*c*): A more superficial plane of focus; arrows mark the vesiculated cellular borders. (*d*): The characteristic "chicken-fence" of the *Stratum corneum*, which overlies the swollen cellular complex. The positioning of the arrows at the outer borders of the darkened granular cells beneath corresponds to that in *c*

Table 2. Electrical changes associated with the cellular swelling which follows impalement of *Str. granulosum* cells with 3-M KCl electrodes[a]

Period	V_e (mV)	R_e/R	$(R_{ip})_t/(R_{ip})_{t_0}$
t_0	-44.01 ± 1.22 (66)	1	1
t_1	-65.23 ± 1.92 (65)	0.48 ± 0.03 (61)	1.53 ± 0.07 (36)
t_2		0.54 ± 0.03 (41)	1.80 ± 0.11 (35)
t_3	-12.76 ± 1.96 (65)	0.45 ± 0.03 (60)	1.41 ± 0.04 (36)

[a] Outside and inside solution Na-sulfate Ringer's. Each entry is an average value ± SEM based on the number of observations in parentheses. Results were obtained from 7 epithelia, using the impalement technique described for Fig. 3. Time intervals correspond to those appearing in Fig. 3, whereby t_0-values give those electrical parameters as measured prior to impalement, i.e., V_e at t_0 is V and R_e at t_0 is R. The increase in R_{ip} observed following impalement is given relative to the value observed prior to impalement (7.12 ± 0.67 (36) MΩ).

Electrical Changes during Swelling

With 3-M KCl electrodes which caused swelling, a large spontaneous change of V_e in the positive direction was observed (period t_2 in Fig. 3 and Table 2). The onset of this change was immediate when swelling was immediate, and was delayed when swelling was delayed. The change was accompanied by a transient increase of R_e and a transient increase of R_{ip} (Fig. 3). In some instances, the change of V_e to positive values occurred in several discrete steps. Results are summarized in Table 2.

Pre-Tip Potentials Observed after Destruction of Plasma Membranes

The potentials recorded from impaled epithelial cells after swelling cannot be representative of unimpaled cells. However, since swelling usually occurs with a delay, it is conceivable that the potentials recorded in period t_1 prior to swelling are meaningful. This would require that V_e in period t_1 is not affected by the onset of KCl leakage from the electrode tip. However, particularly in those epithelial cells of the skin, which contain a high concentration of dense organelles [tonofibrils (prekeratin) and dense mucous granules], it may be suspected that KCl-leakage generates a diffusion potential in a fixed-charge matrix surrounding the electrode tip (compare Davis *et al.*, 1970 [10]). Then, if negative fixed charges dominate and attain a high local density through compres-

sion by the advancing tip, the tip will appear negative with respect to its surroundings.

To investigate this possibility, we recorded intracellularly from epithelia, the membrane potentials of which had been eliminated through mechanical destruction of the cytoplasmic membranes by means of ice crystallization. The preparation was frozen at −20 °C for approximately 10 min and subsequently returned to room temperature. (Transepithelial voltage and resistance were negligible after this treatment. Swelling and change in optical appearance on impalement were not observed in this state.) Impalement of cells was then performed under high gain optical control using K-sulfate Ringer's on both sides of the epithelium. Microelectrodes filled with either 3 M KCl or isotonic K-sulfate Ringer's were employed. Electrode advancement was stopped when the tip was clearly seen to rest in a cell of the outer *Str. granulosum*. R_{ip} was found to increase during advancement. By a slight withdrawal of the electrode R_{ip} could be decreased while maintaining the tip within the cell (compare Fig. 3, label *j*). This withdrawal was purposely not done in the experiments of Fig. 4. Thus, electrode tip pressure on protoplasmic constituents was maintained during recording. If on final complete withdrawal of the electrode R_{ip} was found changed by more than 10% (seldom), the measurement was rejected.

The intracellular potentials (V_e) obtained by this procedure and plotted as a function of the increase in electrode input resistance (ΔR_{ip}) for both electrode fillings appear in Fig. 4. The initial values of R_{ip} were low in the measurements of Fig. 4*A* and higher in those of Fig. 4*B*. It is seen that in both cases negative potentials exceeding −10 mV can be recorded even for relatively small increases in electrode resistance when using 3-M KCl electrodes. In contrast, the potentials clearly remained smaller when isotonic K-Ringer's electrodes were used, and were limited to −1 or −2 mV in those cases where R_{ip} did not increase more than 100%.

Comparison of Fig. 4*A* and *B* shows that the pre-tip potentials recorded with 3-M KCl electrodes tend to be somewhat smaller when the initial input resistance of the electrode is larger. This relationship was investigated more closely by recording with 3-M KCl electrodes of different initial R_{ip} from the same freeze-thawed preparation. In these experiments, after impalement of a cell in the outer *Str. granulosum*, the electrode was withdrawn slightly, such that tissue deformation was partially released and R_{ip} decreased as far as possible while the tip remained within the cell (compare Fig. 3, label *j*). ΔR_{ip} did not exceed

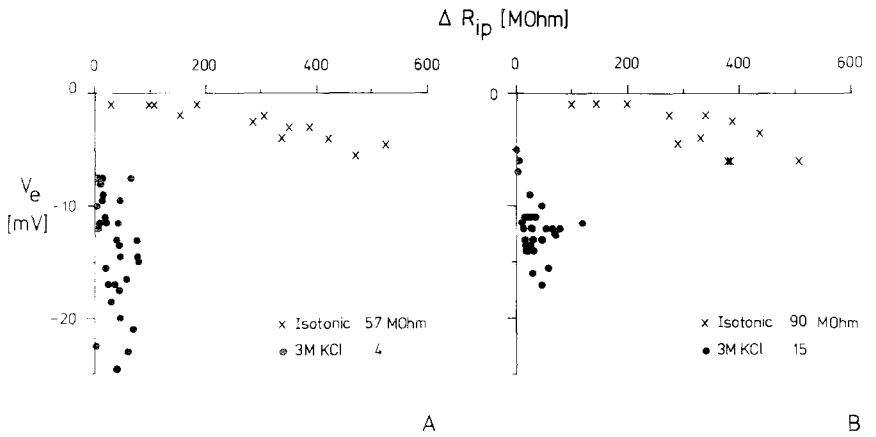

Fig. 4. Microelectrode pre-tip potentials as a function of the increase in electrode resistance on impalement. Plasma membranes were mechanically destructed by ice crystallization during a freeze-thaw cycle (3 epithelia). Impalements of the outer *Str. granulosum* were then performed under microscopic control. Readings were taken without prior release of tissue compression by slight withdrawal of the tip. In each diagram, values are given for one electrode filled with 3 M KCl (circles) and for one electrode of the same tip dimensions filled with isotonic K_2SO_4 Ringer's solution (crosses). The initial electrode resistances (listed) were higher in experiment *B*. Bulk solution: K-sulfate Ringer's

the initial R_{ip} value by more than 20% when a potential reading was taken. In Fig. 5 the pre-tip potentials are plotted as a function of the initial electrode resistance. They clearly become less negative when the initial electrode resistance is larger. (Comparison of Fig. 5 *A* and *B* shows that rather different potential amplitudes are obtained from different epithelial preparations under otherwise identical conditions. It is tempting to explain this variability by different initial concentrations of protoplasmatic charges, which may be characteristic for different phases of the moulting cycle.)

In combination, the results of Figs. 4 and 5 indicate that pre-tip potentials remain small when the 3-M KCl electrode (i) has an initial input resistance above 20 MΩ and (ii) does not compress the protoplasmic material encasing the tip.

Chemical Modification of Fixed Charge Density

The carbodiimide-glycylmethylester reagent (CD) was used to modify (remove) carboxyl-groups from the cytoplasm. Freeze-thawed epithelia

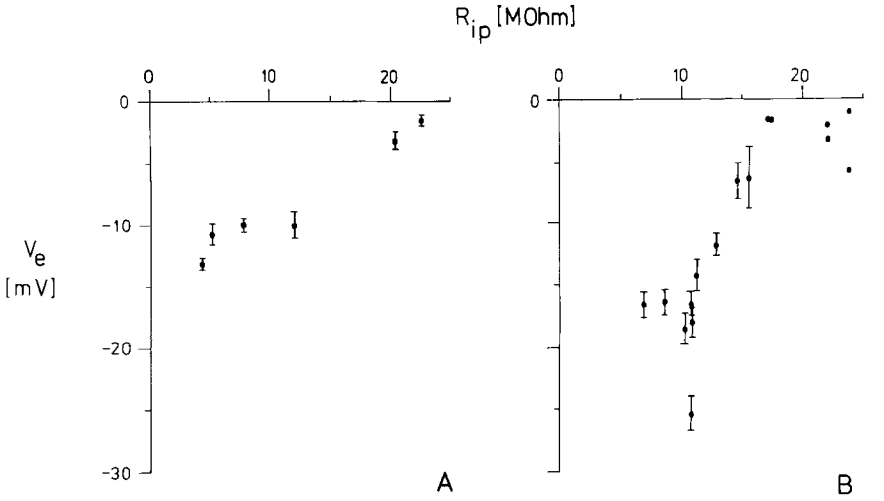

Fig. 5. Pre-tip potentials of 3-M KCl electrodes as a function of the initial electrode resistance measured in cells of the outer *Str. granulosum* of 2 freeze-thawed epithelia (*A* and *B*). Before recording V_e, the electrodes were slightly withdrawn to decrease the tip pressure exerted on protoplasmic constituents as described for Fig. 3. (The resulting decrease in R_{ip} was taken as an indication of the decrease in pressure.) With each electrode between 2 and 20 cells were punctured. Bars indicate SEM

were exposed to this reagent (0.5 M carbodiimide, 1.5 M glycylmethylester, pH 3) for 45 min, then washed with K-Ringer's for 15 min. Subsequent to this treatment, 3-M KCl electrodes recorded positive rather than negative intracellular potentials at pH 7.0 (Figs. 6, 7). It appears that after removal of carboxyl-groups positive fixed charges (presumably amino groups) remain in the cytoplasm and give rise to positive microelectrode potentials if the electrode is filled with 3 M KCl.

A reduction in the density of positive fixed charges remaining after carbodiimide treatment was attempted by exposure of the tissue to ninhydrin, a reagent which desaminates (and decarboxylates) amino acids. Fig. 6 compares potentials obtained after repeated cellular punctures under control conditions, after treatment with carbodiimide, and after subsequent exposure to the CD-treated tissue to ninhydrin. Note the significant reduction of the positive "cellular" potentials which were observed in the carbodiimide-treated tissue, following further tissue exposure to ninhydrin.

The small pre-tip potentials found with electrodes filled with isotonic K-Ringer's were not significantly affected by chemical modification of fixed charge density.

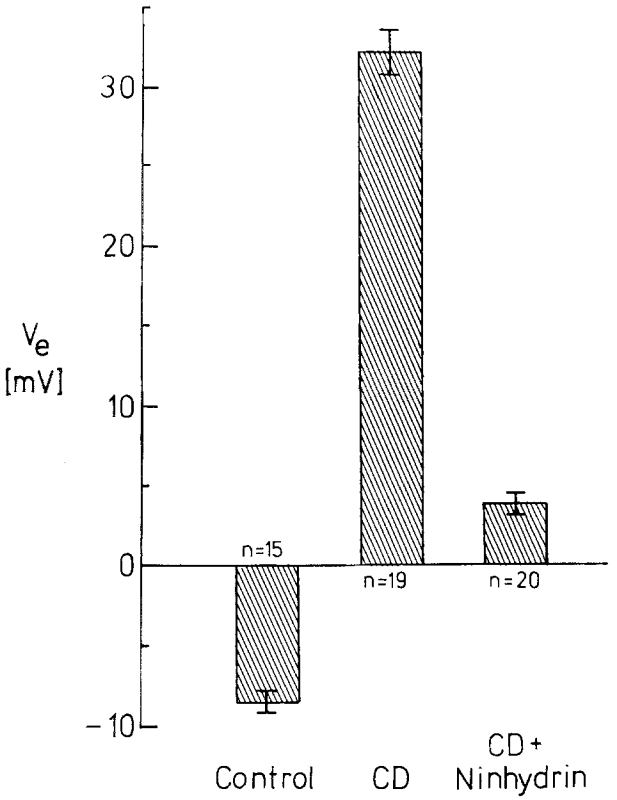

Fig. 6. Comparison of intracellular potentials following treatment of a freeze-thawed epithelium with reagents known to irreversibly remove cytoplasmic fixed charges. Repeated cellular punctures were made under control conditions, after removal of carboxyl groups from the cytoplasm with the carbodiimide reagent (CD), and after further removal of the residual cytoplasmic amino groups with ninhydrin (procedures described in text). Each column represents the mean of repeated cellular punctures, the number of which appears at the base of each column. The vertical bars indicate the SEM. Impalement technique as described for Figs. 3 and 5

Effect of Bulk pH on Pre-Tip Potentials

If protoplasmic fixed charges are instrumental in the generation of pre-tip potentials, it should be possible to affect these potentials by appropriate changes of the bulk pH. In a number of experiments with freeze-thawed epithelia, the ambient pH was decreased by superfusion with K-sulfate Ringer's of pH 3 while maintaining the recording electrode in position. With 3-M KCl electrodes, V_e became less negative and often positive at low pH (Fig. 7*A*), while potentials were small and almost unresponsive to pH changes when electrodes filled with K-sulfate Ringer's

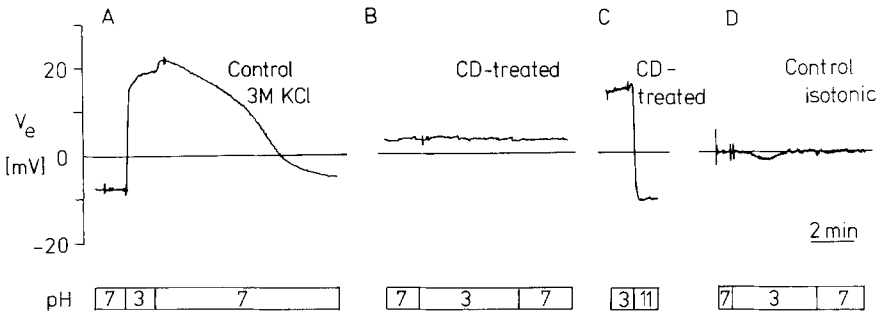

Fig. 7. The effect of bulk pH on the measurement of intracellular potentials following membrane destruction. Impalement technique as described for Figs. 3 and 5. (*A*): Control potential recording after cellular puncture of freeze-thawed epithelia with indwelling 3-M KCl electrode (7.5 MΩ) as superfusion of the tissue is changed from pH 7 to pH 3 and back to pH 7. Note that immediately upon exposure to low pH, V_e became positive. (*B*): Potential recording made with identical indwelling KCl electrode in the same tissue as in (*A*), treated, however, for 45 min with 0.5 M carbodiimide, 1.5 M glycylmethylester (CD) at pH 3, followed by a 15-min superfusion period with K-Ringer's solution at pH 7. Note that now V_e is unresponsive to lowering of the ambient pH. (*C*): Potential recording in similarly CD-treated tissue which was equilibrated in pH 3 prior to raising the ambient pH. Of note here is the abrupt decrease in potential seen when the pH is increased to 11. (*D*): Control potential recording made in untreated freeze-thawed epithelia using a microelectrode filled with isotonic K-sulfate Ringer's solution. Note the absence of a potential while superfusing with a K-Ringer's solution of pH 7, and that the potential is relatively unresponsive to changes in ambient pH

were used (Fig. 7 *D*). Neither type of electrode responded to pH changes while the tip remained in the outer bathing solution. After removal of negative fixed charges with carbodiimide, a pH-step from 7 to 3 did not change the recorded (positive) potential (Fig. 7 *B*), while a change to pH 11 caused a decrease of potential to negative values (Fig. 7 *C*). It should be noted here that both at pH 3 and after carbodiimide treatment the mechanical rigidity of the tissue increased. Nevertheless, the sign of the recorded potentials was reversed under these conditions. These results support our conclusion that the pre-tip potentials depend on sign and density of protoplasmic fixed charges.

The results so far reported illustrate that "pre-tip potentials" are artifactual in nature in that they are obtainable under conditions where membrane potentials are absent and are dependent (i) on sign (pH, chemicals) and density (tip pressure) of fixed charges present in the cellular cytoplasm as well as (ii) on an electrolyte gradient (electrode filling, electrode tip resistance) in the organic matrix encasing the tip.

Relationship between Pre-Tip Potentials and Input Resistance during Tip movement

The magnitude of pre-tip potentials (V_e) recorded from freeze-thawed epithelia was found to be somewhat arbitrary in that it could be increased by a slight advancement or decreased by a slight withdrawal of the microelectrode. Concomitant changes of input resistance (R_{ip}) were always noted and taken as a rough indication of the degree of compression of organic material by the tip. The effect of tip movement on V_e and R_{ip} was studied systematically with 3-M KCl electrodes of initial resistances above 20 MΩ as shown in Figs. 8 and 9. The recording of Fig. 8 *D* was obtained with a tip filled with 3 M KCl of pH 7. The initial tip resistance was 28 MΩ. Bathing solution was K-sulfate Ringer's of pH 7. The tip was first pushed into a cell of the outer *Str. granulosum* of a freeze-thawed epithelium (label i), then withdrawn slightly (as described for Figs. 3 and 5) to counteract the changes of V_e and R_{ip} which occured during impalement. At this stage V_e was -2 mV and R_{ip} was 30 MΩ, showing that pre-tip potentials of 3 M KCl electrodes can be negligible if the electrode is of high initial resistance and does not exert pressure on protoplasmic constituents (*compare* Fig. 5).

During subsequent slow advancement of the electrode, R_{ip} increased and V_e became negative. When R_{ip} had reached 180 MΩ, the electrode was slowly withdrawn to its starting position within the cell. In response, R_{ip} and V_e returned to their starting values. During these changes, R_{ip} and V_e were also plotted against one another using an X–Y recorder (lower diagram). This plot shows clearly that on slow advancement of the electrode there was first a large change of V_e accompanied by an only moderate increase of R_{ip}. On further advancement, V_e did not change much but R_{ip} increased steeply. On slow withdrawal of the electrode, the sequence of events reversed. The resulting X–Y trace was indistinguishable from that obtained during advancement. On final withdrawal of the electrode from the cell (label *r*), V_e and R_{ip} attained their initial values.

These results with 3-M KCl electrodes of resistances above 20 MΩ show that pre-tip potentials develop as soon as protoplasmic material is compressed by the tip. However, the resulting increase of R_{ip} will not necessarily warn against the development of a pre-tip potential, since the major change in R_{ip} occurs after the major change in V_e. The reversibility of the increase in R_{ip} indicates that the resistance increase is indeed caused by compression of material in front of the tip rather

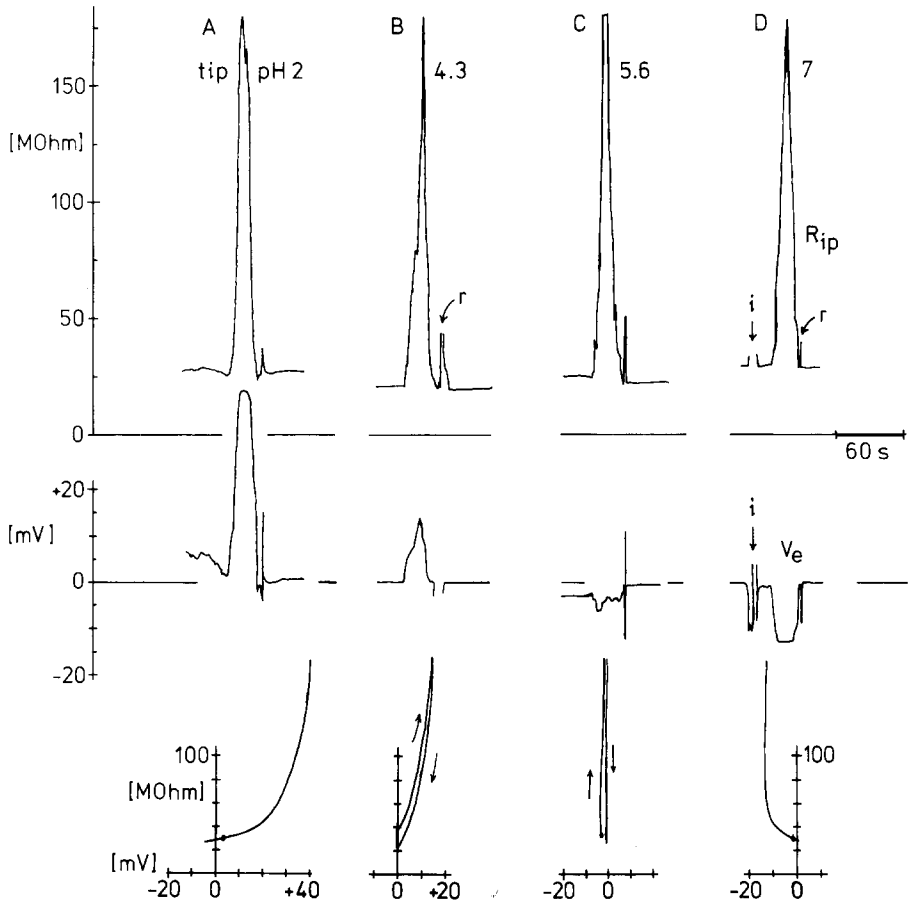

Fig. 8. Effect of tip-pH on pre-tip potentials obtained during electrode movement. Simultaneous recordings of R_{ip} and V_e were done during slow advancement and subsequent withdrawal of the electrode tip, which had been placed into a cell of the outer *Str. granulosum* under microscopic control. Freeze-thawed preparation. Initial electrode resistances were above 20 MΩ. *i*: impalement artifact; *r*: artifact due to complete withdrawal of electrode. Bulk solution: K-sulfate Ringer's of pH 7. Tip filling: 3 M KCl of the pH value indicated (0.1 M citric acid-phosphate buffer). The lower diagrams show R_{ip} as a function of V_e. Sign and amplitude of the movement-dependent change in V_e is affected by tip-pH. The change in R_{ip} is not affected

than by a filling of the tip with organic material. An irreversible increase in R_{ip}, explainable by plugging of the tip interior with protoplasmic constituents, occurred rather infrequently.

Dependence of the V_e–R_{ip} Relationship on Tip pH

At neutral bulk pH, pre-tip potentials were found to be strongly affected by decreasing the pH of the solution within the microelectrode.

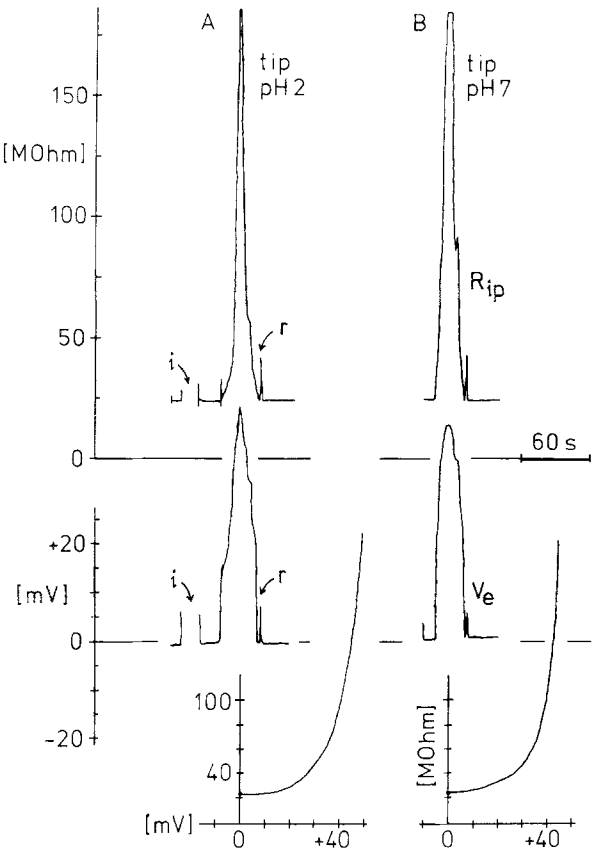

Fig. 9. An increase of tip-pH is without effect on pre-tip potentials when bulk pH < tip pH. Preparation and electrode movement as described for Fig. 8. Bulk solution: K-sulfate Ringer's of pH 2. Tip filling: 3 M KCl of pH 2 (*A*) and pH 7 (*B*)

In the experiment of Fig. 8, 3 M KCl buffered to pH 7, 5.6, 4.3, and 2 was used to fill the electrodes. The bulk solution was K-sulfate Ringer's of pH 7. It can be seen that, irrespective of tip-pH, the input resistance increased on advancement and decreased on withdrawal of the microelectrode. In contrast, the pre-tip potential became negative on advancement when the tip-pH was above 5.6, but positive on advancement when the tip-pH was below 5.6. Tips of pH 5.6 showed little change of V_e on advancement.

Titration of fixed charges by electrode pH is not effective if the bulk pH is lower than that of the electrode. In the experiment of Fig. 9, the bulk solution was K-sulfate Ringer's buffered to pH 2. The microelectrode was filled with 3 M KCl buffered to pH 2 or pH 7. Regardless of electrode filling, V_e became positive on advancement. Both resulting

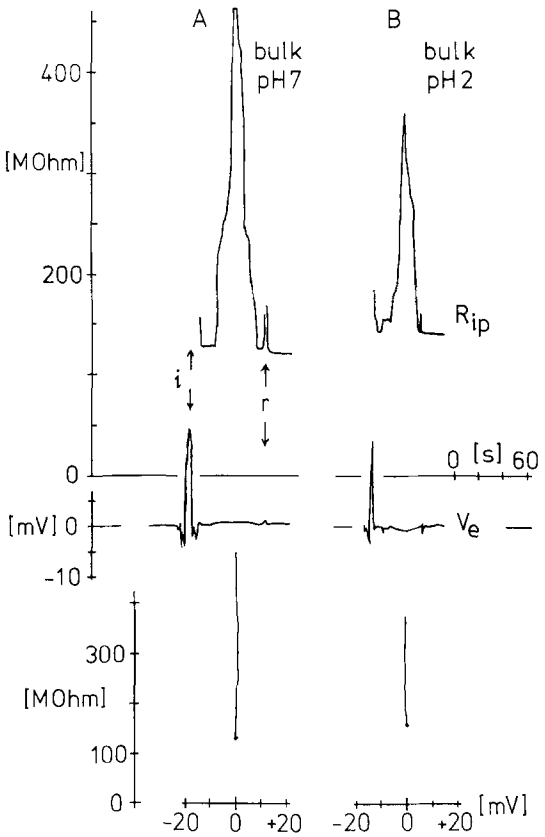

Fig. 10. When using tips filled with isotonic K-sulfate Ringer's (pH 7), V_e does not change during slow tip movement but R_{ip} does. This behavior is observed both at neutral and acid bulk pH. Preparation and electrode movement as described for Fig. 8. Bulk solution: K-sulfate Ringer's of pH 7 (*A*) and pH 2 (*B*)

V_e *vs.* R_{ip} plots showed the pattern of Fig. 8*A*, where the bulk pH was 7 and the tip-pH was 2. Thus, as may be expected, the solution with the higher proton concentration – be it the bulk phase or the tip filling – dominates in affecting fixed charges and pre-tip potentials.

In conclusion, sign and density of fixed charges in front of the electrode tip can be affected not only by the bulk pH but also by the pH of the electrode filling, if the electrode pH is lower than the bulk pH. The change in R_{ip} is not clearly affected by titration and, therefore, is not likely to depend on fixed charges.

We have implied above – following Davis *et al.* [10] – that fixed charges in the protoplasmic space surrounding the electrode tip will permit the development of a KCl diffusion potential if the tip contains a high concentration of KCl (*see also Discussion*). Like Davis *et al.* [10]

we found support for the importance of a local KCl-gradient in experiments where the tip was filled with isotonic saline. In the experiment of Fig. 10 the microelectrode contained K-sulfate Ringer's buffered to pH 7. The bulk solution was K-sulfate Ringer's of pH 7 or pH 2. It was noted that during impalement (*i*) the V_e-artifact was smaller than usually seen with 3 M KCl electrodes. On advancement and withdrawal R_{ip} changed in the familiar way but V_e remained constant both at pH 7 and at pH 2. In consequence, the V_e *vs.* R_{ip} plots became vertical lines.

This result confirms our previous conclusion that pre-tip potentials can be made negligible by avoiding large electrolyte gradients between electrode and cytosol. Titration of fixed charges is then without effect. Movement-dependent changes of R_{ip} are maintained.

Discussion

The Computed Rate of KCl-Loss

Glass microelectrodes are filled with 3 M KCl in order to keep tip resistance and tip diffusion potentials small. It was realized early that such electrodes lose KCl. Nastuk and Hodgkin [32] speculated that a tip of 0.25 μm inner diameter filled with 3 M KCl and having a resistance of 20 MΩ will lose KCl at a maximal rate of 0.06 pmole/sec. Coombs, Eccles and Fatt [9] computed in more detail that an electrode of 10 MΩ resistance will lose at most 0.04 and one of 5 MΩ resistance at most 0.06 pmole KCl/sec. Recent extensive computations by Geisler *et al.* [14, *see also* 13] confirmed these predictions. In view of this agreement it is justified to assume that our 5 MΩ electrodes lost roughly 0.06 pmole KCl/sec. This amounts to 0.12 posmol/sec and constitutes a considerable osmotic load for an epithelial cell with a volume of 500 μm^3 and an initial osmotic content of about 0.1 posmole. The observation of cellular swelling, therefore, is not surprising. Assuming, as did Coombs *et al.* [9], that the KCl loss is roughly proportional to electrode conductance, we may predict that an electrode of 20 MΩ resistance will lose about 0.015 pmole KCl/sec if the tip is not obstructed. This lower rate of loss should still result in a noticeable osmotic load. The KCl loss from very small tips may be less than predicted from electrode conductance, if with decreasing tip diameter the ion exchange properties of the glass wall become more dominant (Küchler [19], Lavallée and Szabo [22]). To confirm this, it would be desirable to obtain direct measurements

of the KCl diffusion from electrodes of high and low resistance. In the absence of such data, we shall presently rely on the computed rates of KCl loss.

Osmotic Effects of KCl Injection

The expected somewhat lower rate of KCl loss from 20 MΩ electrodes may be negligible if the electrode impales a large cell, for example, a striated muscle cell. However, when small cells like erythrocytes, Ehrlich ascites tumor cells, etc., are impaled, their composition will change rather quickly even by the addition of only 0.006 pmole KCl/sec. From this rate it can be predicted that a cell of 1000 μm^3 volume will double its osmotic content in the first 20 sec after impalement. Osmotic water uptake, as well as diffusional loss of KCl through the plasmalemma, through the "impalement shunt" [23], and through cell junctions will counteract the increase of osmolarity to some extent. On the whole, however, the cellular potential measured cannot be expected to be representative, except perhaps when extrapolated back to the time of impalement [21]. In those epithelial cells which are small but coupled by gap junctions, the amount of KCl "injected" by diffusion may distribute in a volume much larger than that of a single cell. However, distribution from cell to cell by diffusion requires a gradient. Therefore, an impaled, coupled cell must nevertheless be expected to undergo a significant increase in its K- and Cl-concentration.

Swelling does not occur with electrodes filled with isotonic K-sulfate. This excludes the possibility that a stimulation of transport, caused by a depolarization of the impaled membrane by means of an impalement shunt, will produce the increase in cell volume. Bulk-flow and current-flow through the electrode tip have also been excluded as being instrumental in the swelling process, leaving KCl diffusion from the electrode tip, and a subsequent increase in osmotic content and volume of the impaled cell to explain the swelling phenomenon. If the electrode tip were to deliver KCl continuously, swelling might be expected to start immediately following impalement. However, we observed that in many instances swelling began after a delay of 10–100 sec. The slow removal of tip-obstructing tissue particles, a diffusion hindrance in the space surrounding the tip and/or delayed closure of an impalement shunt, may explain this time course (*see below*).

Swelling is not restricted to the impaled cell but is usually shared

by several neighboring cells. At first glance, this observation seems to indicate that the cells are normally coupled through low-resistance diffusion pathways (gap junctions). However, this conclusion cannot be accepted without reserve, because (i) coupling could develop during the swelling process and (ii) KCl could reach the neighboring cells through the interstitial space. In this context it is interesting to note that the interstitial space between and around the swollen cells appears enlarged (Fig. 1 and 2). Quite conceivably, the laterobasal membranes of the swollen cells, which in their normal state are known to have a high K and Cl permeability [18], permit diffusion of excess KCl into the interstitial space, which would then increase its volume by osmosis.

In intentional imitation of most other groups previously working with amphibian skin, we used electrodes with resistances below 20 MΩ for the swelling experiments. The temptation to use such electrodes is large because they break less frequently when forced through the rigid *Str. corneum.* As shown in Table 1, 16 out of 17 groups facing this problem have employed such electrodes. Cereijido and Curran, 1965, used 3-M KCl electrodes between 1 and 5 MΩ [6], Nunes and Lacaz Vieira, 1975, those of 1–10 MΩ [33], and Nagel, 1975 to 1977, 5–9 MΩ resistance [26–31]. It must be suspected that all these electrodes lost KCl rapidly. However, even the less conspicuous KCl-loss from 20 MΩ electrodes may be expected to increase the cellular K and Cl concentration of small cells considerably. This increase will be more significant when slow sealing of the impalement shunt calls for keeping the electrode within the cell for a long time. On the whole, therefore, the ill-advised use of low resistance electrodes only aggravates a problem which also exists with smaller electrode tips filled with 3 M KCl.

While tips of more than 20 MΩ resistance might lose KCl at a rate which is not sufficient to cause visible osmotic swelling, this loss can still result in certain electro-chemical artifacts (pre-tip potentials) which are discussed below. To avoid KCl-leakage, extremely thin 3 M KCl electrodes or electrodes filled with isotonic salt solution may be used. With such electrodes, too, the contribution of tip potentials to the recorded potentials must be carefully evaluated.

Changes of Electrical Parameters during Swelling

The osmotic changes and the electrical changes which accompany them are certainly artifacts. It may, therefore, not be of great interest

to explain the electrical events observed during swelling in detail. Nevertheless, we shall attempt to offer a reasonable explanation which also covers the interesting fact that swelling does, in most cases, develop with a delay.

It is likely that right after impalement the apical membrane potential will be partially short-circuited by the impalement shunt [23]. Thus, much of the KCl entering the cell through the "pipette" may leave by this route. In this case, swelling would develop slowly at first. When later on the impalement shunt closes more tightly, possibly in response to the initial swelling, faster swelling would become possible. As mentioned above, a slow removal or loosening of protoplasmic material which plugs the tip or constitutes the fixed charge space in front of the tip may contribute to the delay.

During swelling, V_e increased on the average by 52 mV to values which were positive with respect to the outside solution and negative by 13 mV with respect to the inside solution. At the same time, R_e increased transiently by 10% and R_{ip} increased transiently by 6 MΩ (on the average). Two mechanisms which may contribute jointly to these electrical changes are mentioned as hypothesis:

1) Due to osmotic uptake of water, the fixed-charge density around the microelectrode tip may decrease. At the same time, the KCl gradient around the tip will diminish as KCl leakage through the impalement shunt decreases. The KCl diffusion potential within the cytosol will, thus, diminish or disappear. As a result V_e becomes less negative during swelling.

2) If the tip loses KCl rapidly, a KCl-diffusion potential at the K-permeable latero-basal membrane can be expected to develop and, as mentioned before, contribute to the initial negative value of V_e. In the course of some seconds an increase of the interstitial KCl-concentration may occur, and decrease the KCl gradient across the latero-basal membrane. The diminished gradient, and particularly a change of membrane properties during swelling, might be responsible for the delayed change of V_e to more positive values.

The cellular potential recorded after swelling will be some mean of the potentials of the two bathing solutions. Its value will depend on the alterations of permeabilities, i.e., the extent of damage which has occurred at the apical and latero-basal membranes. The transient changes of R_e and R_{ip}, which are observed as V_e increases, may reflect these alterations of permeabilities at apical and latero-basal membranes in response to cellular swelling.

In the past, the stability of a recorded cellular potential has often been taken as a sign of reliability. It is noteworthy, therefore, that the value of V_e observed after swelling, which is generally positive with respect to the outer solution, is stable. If no notice were taken of the KCl-leakage and the swelling phenomenon, one would describe that after impalement an unstable potential negative to the outer solution is observed, followed by a stable and, therefore, reliable potential which is positive to the outer solution. On the other hand, if the impalement technique causes much mechanical strain and, thus, tissue compression in front of the electrode tip, it is likely that swelling occurs only occasionally. Then stable potentials negative to the outer solution may be reported which show only infrequent spontaneous changes in the positive direction.

The spontaneous change of V_e reported in this paper occurs both in the presence and absence of Na in the outer solution. In contrast, we found that with isotonic microelectrodes another kind of spontaneous potential change can be recorded, which is reversible and apparently depends on the absence of Na in the outer solution. It will be described in a subsequent publication.

Intracellular Potentials Recorded with 3-M KCl Electrodes Prior to Swelling

The initial negative value of V_e, observed before rapid swelling begins, poses a problem. Granular cells are claimed to be permeable to Na at their apical membrane and permeable to K at their latero-basal membrane [18]. Thus, given open-circuit conditions and the presence of Na as well as the absence of permeant anions in the outer solution, a positive potential step is expected when the tip passes through the apical membrane. However, a "negative step" was observed. The same observation has been reported for example by Cereijido and Curran [6], by Nagel [26–31], and by Helman and Fisher [15]. (However, as these groups may have recorded from layers below the outer *Str. granulosum*, the agreement may be coincidental.) As summarized in Table 2, we found V_e, recorded from the outer *Str. granulosum* at time t_1 (i.e., immediately following impalement) to be on the average negative to the outer bathing solution by 21 mV. In the same period, t_1, R_e indicated that the tip had passed at least half of the transepithelial resistance.

A partial explanation for the "negative step" may be found in an argument advanced by Davis, Jackson, Day, Shoemaker and Rehm [10]. These authors reported that 3-M KCl electrodes, when placed into the

corneal stroma of the eye, record potentials of up to 25 mV (tip negative *vs.* aqueous humor). They considered the potentials of −25 mV to be an artifact explained by the diffusion of KCl from the electrode tip through the stroma, which contains negative fixed charges. Electrodes filled with Ringer's recorded zero mV when placed into the stroma. Nunes and Lacaz Vieira [33] have advanced the same explanation for the negative potential of −26 mV recorded with 3-M KCl electrodes in the region of the *Str. corneum* of toad skin. Indeed, there is a certain similarity between the corneal stroma of the eye and the *Str. corneum* and *Str. granulosum* of the skin. In both cases structured protein is abundant (collagen fibres in the corneal stroma and tonofibrillar pre-keratin in the skin), and in both cases acid mucopolysaccharides are also present in large amounts.

The amino-acid composition of keratinlike proteins derived from adult amphibian skin (*Xenopus laevis*) has been reported by Reeves [36]. It appears that the dicarboxy monoamino acid content is larger (14%) than that of diamino-monocarboxy amino acids (4%). Therefore, even when disregarding other proteins and the acid mucopolysaccharides, a net negative charge may be expected at neutral pH. The pH-dependence of KCl diffusion potentials observed with dead frog skin [1] and with the isolated *Str. corneum* of the toad [20] also shows that negative fixed charges dominate at neutral pH. It is reasonable, therefore, to suspect that the fixed charges of epidermal macromolecules are at least in part responsible for the negative potentials recorded with 3-M KCl electrodes in period t_1.

The intracellular potentials obtained with 3-M KCl electrodes from freeze-thawed epithelia and their response to pH changes and to carbodiimide and ninhydrin treatment support this hypothesis. Apparently, protoplasmic fixed charges can give rise to pre-tip potentials which are almost as negative as the mean potentials recorded from intact cells of the *Str. granulosum* prior to swelling. However, it is not certain that the apparent negative potentials of intact epidermal cells are completely explained by fixed charges, due to the fact that the protoplasmic charge density of normal and freeze-thawed cells may not be strictly comparable. Crystal formation during freeze-thawing may be expected to "vacuolize" the protoplasm and, thus, cause local concentration of fixed charges. It may also cause rupture of protoplasmic organelles as well as change the mechanical resistance which facilitates the concentration of fixed charges in front of the tip during electrode advancement. Low pH and carbodiimide was noted to increase mechanical stiffness of the tissue.

Therefore, protoplasmic properties were certainly somewhat disturbed in these "control" experiments. Nevertheless, it is clear that the effect of fixed charges constitutes a serious source of error when recording from granular cells with 3-M KCl electrodes. Evaluation of Figs. 4 and 5 shows that the V_e values given in Table 2, which were obtained with 7-MΩ electrodes, are too negative by at least 10 mV due to pre-tip potentials.

In this paper the protoplasmic fixed charge potential obtained with 3-M KCl electrodes is called "pre-tip" potential in contradistinction to tip potentials recorded in free solution. The name would be particularly appropriate if the hypothesis by Davis *et al.* were correct which explains the pre-tip potential as a KCl diffusion potential in the protoplasmic space bordering at the tip [10]. At a first glance, support for their hypothesis appears to arise from our findings that pre-tip potentials are strongly dependent on local tip pressure and on tip-pH. Particularly suggestive may be the observation that with tips of pH 2 the pre-tip potential becomes positive and is unaffected by pH-changes of the bathing solution. However, these results do not constitute proof. It is equally possible that the pre-tip potentials arise for instance at the interface between bulk solution and organic matrix. At tips of isotonic electrodes an opposite potential step would make the potential profile of the organic matrix symmetrical and, therefore, undetectable. High KCl-concentrations in conjunction with tip pressure may partly abolish this second step and, thus, permit recording of the protoplasmic Donnan potential.

In granular cells it may be the combination of mechanical rigidity (tonofibrils) and a high density of negative fixed charges (tonofibrils plus mucopolysaccharides) which facilitates the recording of pre-tip potentials. The local fixed charge density may be expected to increase as the advancing tip packs protoplasmic constituents tightly against a meshwork of tonofibrils. Rupture of mucous granules is likely to occur, setting free acid mucopolysaccharides.

Returning to the KCl-diffusion potential hypothesis, it may be noted that the transference number of K diffusing from the tip will increase with increasing local density of negative charges. In the limiting (unlikely) case of very high negative-charge density, where the local transference number of K approaches unity, the Nernst potential of K (at room temperature about −80 mV) would develop across the fixed charge layer engulfing the tip. We have not observed pre-tip potentials of this magnitude. The largest values obtained were +50 mV (pH 2).

KCl will not leave the tip area when the transference number of

Cl approaches zero. In this case cellular swelling will not occur. In general, one might expect swelling to be more rapid when the negative pre-tip potential is small. Note, furthermore, that the pre-tip potential is additive to the membrane potentials. Therefore, the total potential recorded by the microelectrode can still respond to changes of membrane potentials effected, for instance, by changes of the outer Na-concentration.

3-M KCl electrodes have often been used in the study of epithelial transport. In the study of frog and toad skin epithelium, of the 27 investigations which employed microelectrodes, all were carried out using 2 to 3-M KCl fillings, all but one were carried out at least in part with tips of less than 20 MΩ resistance, and 8 investigations used tips of resistances below 5 MΩ (Table 1). Because of the osmotic and pre-tip potential artifacts caused by such electrodes, it appears necessary to re-investigate the membrane potentials of amphibian skin epithelium with more suitable techniques.

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