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# Intracellular Ionic Activities in Frog Skin

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**Summary.** Intracellular $Na^+$, $K^+$, and $Cl^-$ activities ($a^i_{Na}$, $a^i_K$, $a^i_{Cl}$) and transapical membrane potentials ($V_o$) were measured with liquid ion-exchanger and open-tip microelectrodes in isolated short-circuited frog skins (*R. pipiens*) incubated at 23 °C in normal amphibian Ringer's solution. Under control conditions $a^i_{Na} = 14 \pm 3$ mM, $a^i_K = 132 \pm 10$ mM and $a^i_{Cl} = 18 \pm 3$ mM (SD). The value of $a^i_{Cl}$ is 4.4 times the value corresponding to electrochemical equilibrium for this ion. Thus, $Cl^-$ is actively accumulated by epithelial cells of the frog skin. Shortly after addition of amiloride (2–5 μM) to the apical bathing medium, $a^i_K$, $a^i_{Na}$, and $a^i_{Cl}$ were essentially unchanged although $V_o$ had hyperpolarized by about 30–40 mV. During long-term exposure to amiloride $a^i_K$ and $a^i_{Cl}$ did not change significantly, $V_o$ depolarized by about 16 mV from the maximal value and $a^i_{Na}$ decreased to $8 \pm 3$ mM. Immediately after exposure to amiloride the transmembrane driving force for $Na^+$ increased from 124 to 154 mV. During further exposure to amiloride, despite changes in both $V_o$ and $a^i_{Na}$, this driving force remained virtually constant. Since $I_{sc}$ during this period was close to zero, it is suggested that the observed driving force for $Na^+$ under these conditions approximates the maximal driving force generated by the $Na^+ - K^+$ ATP-ase pump in the basolateral cell membrane.

**Key words:** Frog skin, microelectrodes, membrane potentials, intracellular activities, amiloride.

Measurements of intracellular ionic activities are required for the accurate assessment of transmembrane electrochemical potential gradients and the energetic evaluation of ion transport across cell membranes. In epithelial systems a knowledge of these parameters is an essential step in understanding the mechanism of transepithelial electrolyte transfer. For this reason, there has been, in recent years, an increasing interest in the measurement, with appropriate ion-selective microelectrodes, of intracellular ionic activities (particularly $K^+$, $Na^+$, and $Cl^-$ activities) in a variety of epithelia.

During the past few years techniques have been developed that permit improved recording, with microelectrodes, of intracellular potentials in epithelial systems. However, these techniques are not free of uncertainties. Microelectrode artifacts are a well-documented phenomenon, particularly in epithelia [13, 15, 19, 21, 25]. Therefore, criteria that permit unequivocal identification of impalement artifacts are essential. In frog skin "specific" criteria for successful microelectrode impalements have been established [13, 23]. In the present study, these criteria, which are discussed in detail below, were applied to the measurement of transapical potentials ($V_o$) and of intracellular $Na^+$, $K^+$, and $Cl^-$ activities ($a^i_{Na}$, $a^i_K$, $a^i_{Cl}$) in epithelial cells of the short-circuited frog skin.

## Materials and Methods

Isolated segments of the abdominal skin of *Rana pipiens* were used in these experiments. Skin segments were mounted, at room temperature (23 °C) in a divided Lucite chamber as described elsewhere [23]. Both sides of the tissue were perfused separately with an aerated Ringer's solution containing, in mM: NaCl, 110; $KHCO_3$, 2.5; $CaCl_2$, 1.0; the pH was 8.1. A special 3-way valve permitted the solution bathing the epithelial side of the tissue to be changed rapidly without interrupting perfusion. The time for complete exchange of the epithelial chamber volume was less than 10 sec.

Transepithelial PD was measured with calomel half cells connected to the solutions by 3 M KCl-agar bridges. Transepithelial current was applied with two AgCl coated silver plates. During the experiments the skins were short circuited except for brief

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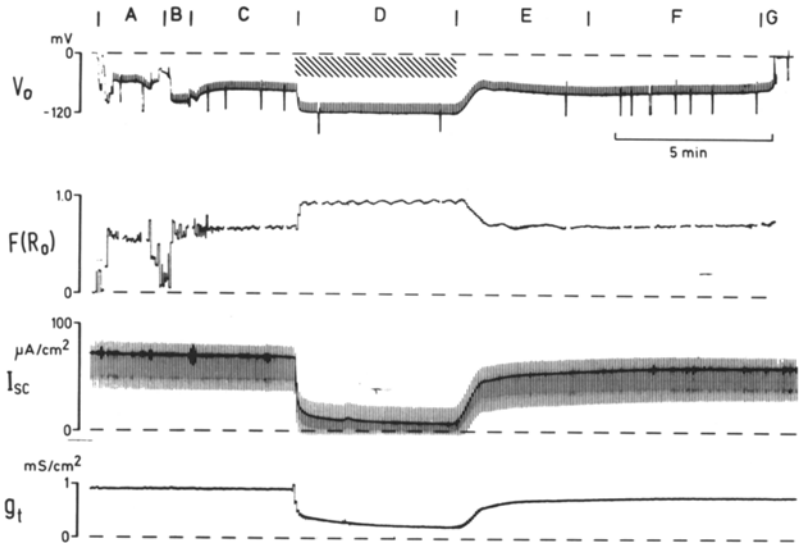


**Fig. 1.** Simultaneous recordings of $V_o$, $F(R_o)$, $I_{sc}$, and $g_t$ in frog skin epithelium. The hatched bar indicates the period during which amiloride (5 μM) was applied. Upward deflections reflect repetitive clamping of transepithelial PD to 10 mV. Downward deflections show manually applied single-current pulses through the microelectrode, (*see* Materials and Methods)

periods (150 msec) during which the transepithelial PD was clamped to 10 mV (corial side positive). The resulting change in transepithelial current was used to calculate tissue conductance ($g$). Intracellular potentials were measured with reference to the epithelial bathing solution. The fractional resistance, $F$ ($R_o$), of the apical membrane was calculated from the potential change across this membrane, $\Delta V_o$, when the transepithelial PD was clamped to 10 mV. Short-circuit current ($I_{sc}$), $V_o$, $F(R_o)$, and $g_t$ were recorded on four channels of a strip chart recorder (Gould-Brush, Mark 240, Cleveland, Ohio). In addition, the numerical values of these parameters were displayed on digital panel meters and these values were used to calculate potentials and ionic activities. The fabrication, calibration, and characteristics of the open-tip and $Na^+$, $K^+$ and $Cl^-$ – selective microelectrodes used in this study were as previously described [11]. Microelectrodes were connected through Ag/AgCl wires and a guarded coaxial cable to a high impedance ($>10^{14}\Omega$) preamplifier (Analog Devices 311 J, Norwood, Mass.) connected in series to a differential amplifier (Burr Brown, Inst. Amplif. 3621 K, Tucson, Arizona). Input impedances of open-tip microelectrodes were measured by the deflections produced by a $10^{-9}$ A current pulse. Open-tip microelectrodes were filled with 1.5 M KCl and had resistances between 20 and 40 MΩ when the tips were immersed in Ringer's solution. The microelectrodes were advanced perpendicularly to the epithelial layer using a fast stepping-motor micromanipulator (Nano-stepper, Marchinowski, Heidelberg, Germany). Specific details of the acceptability of impalements are as previously described [21, 23] and as discussed below. A minimum of four acceptable impalements was made with each conventional and ion-selective microelectrode. Measurements of $V_o$ with open-tip microelectrodes were made before and after impalements with ion-selective microelectrodes. Intracellular ionic activities were calculated by reading directly from the calibration curve the value of the intracellular electrode potential after correction for the mean $V_o$, as described in detail elsewhere [3, 11]. The $Na^+$-selective microelectrodes were calibrated in NaCl solutions containing a constant $K^+$ concentration of 200 mM. This approximates the mean $a_K^i$ measured in the present experiments.

## Results

Recordings were made with 11 skins. During the recording period, these skins had control short-circuit currents between 16 and 76 μA/cm². The average value was 43±7 (SEM) μA/cm². Tissue conductance ranged from 0.5 to 2.4 mS/cm² with a mean value of 1.3±0.2 mS/cm². After the addition of 2–5 μM amiloride[1], the average short-circuit current decreased to 6±1 μA/cm² and $g_t$ decreased to 0.53± 0.08 mS/cm². These control values, and the values observed in the presence of amiloride, are typical for frog skins mounted in Ussing type chambers and perfused on both sides with Ringer's solutions [13, 21, 23].

Figure 1 (segments *C, D,* and *E* of the top tracing) is the original record of an open-tip microelectrode impalement that was considered acceptable. Prior to this (segment *A*) an impalement was obtained that was not considered to be acceptable for two reasons. First, the membrane potential did not reach a stable value. Second, during this segment of the recording, $F(R_o)$ was variable and low. On further advancement of the microelectrode at the same site (segment *B*) there was a sharp deflection in the membrane potential followed by the establishment of the steady-state value of −91 mV. However, at this time the microelectrode resistance increased from 31 to >200 MΩ (off scale pulse) indicating that the microelectrode tip was partially obstructed. When the microelectrode was slightly withdrawn (segment *C*) the membrane potential fell to between −67 and −69 mV and the

[1] Amiloride was used at a concentration of 2–5 μM, rather than at higher concentrations that would decrease the short-circuit current to lower values. This was done because we observed that amiloride, in concentrations greater than 10 μM, significantly affected the potentials registered by $K^+$-selective microelectrodes in the Ringer's solution used in these experiments. With 2 μM amiloride, this change in potential was less than 0.5 mV. This is well within the range of experimental error in determining $K^+$ activities with these microelectrodes.

microelectrode resistance decreased to 38 MΩ. However, the average value of $F(R_o)$ did not change between periods *B* and *C* of Fig. 1, indicating that there was no significant membrane leakage around the microelectrode tip. Addition of 5 μM amiloride (segment *D*) was followed by typical decreases in $I_{sc}$ and $g_t$. In addition, there was a rapid increase in $V_o$ to −111 mV. Simultaneously, $F(R_o)$ increased from 0.68 to 0.95. The microelectrode resistance remained virtually constant. Removal of amiloride (segment *E*) was followed by recovery of $I_{sc}$ and an increase in $g_t$. At the same time $V_o$ depolarized rapidly to −64 mV. This was followed by a relatively slow repolarization to −75 mV. During this time microelectrode resistance remained constant and $F(R_o)$ decreased to its initial value.

As a further test of the validity of the values recorded during periods *C*, *D*, and *E* of Fig. 1, the microelectrode was further withdrawn in steps of 1 μm (segment *F*). Note that $V_o$ decreased only slightly (to −71 mV) and $F(R_o)$ and microelectrode resistance remained virtually constant until the microelectrode was withdrawn almost completely from the cell. Finally, when the microelectrode was withdrawn completely from the cell, $V_o$ returned rapidly to a value that differed by only −2 mV from its initial level.

In some experiments, unusually high values of $V_o$, both before and after exposure of the tissue to amiloride, were occasionally observed at relatively low values of the microelectrode resistance (i.e., not more than 5–10 MΩ above the value in free solution). These values, which ranged as high as −140 mV in the presence of amiloride, occurred in a random fashion in individual impalements and were rather rare (i.e., not more than 5% of the total impalements recorded). They were disregarded if, as in fact invariably occurred, lower values of $V_o$ that displayed the same response to amiloride were recorded with the same skin. Finally, it should be noted that, with these exceptions, $V_o$, $F(R_o)$ and their response to amiloride did not vary significantly throughout any given experiment.

### *Recordings with $Cl^-$-Selective Microelectrodes*[2]

Figure 2 shows the intracellular potential recorded with a $Cl^-$-selective microelectrode ($V_{Cl}$) together with a simultaneous recording of $I_{sc}$. A tracing of $V_o$ made subsequently on the same skin is also shown. In these recordings, the cell was impaled following 25 min exposure to 5 μM amiloride. The steady-state potential differences after impalement were −87 mV for $V_o$ and −65 mV for $V_{Cl}$. $F(R_o)$ was 0.95 for the open-tip recording and 0.93 for the recording with the $Cl^-$-selective microelectrode. Following removal of amiloride, the maximal depolarizations ($\Delta V_o$ and $\Delta V_{Cl}$) were 30 and 28 mV, respectively. In both cases, depolarization was followed by a gradual repolarization. During 3 min under amiloride-free conditions, $V_o$ repolarized by 11 mV and $V_{Cl}$ repolarized by 9 mV. When amiloride was re-admitted to the mucosal solution, $V_o$ and $V_{Cl}$ assumed values that were, respectively, 9 and 7 mV more negative than their initial values in the presence of this agent. However, the total changes in potential following addition of amiloride (28 and 25 mV, respectively) were again similar.

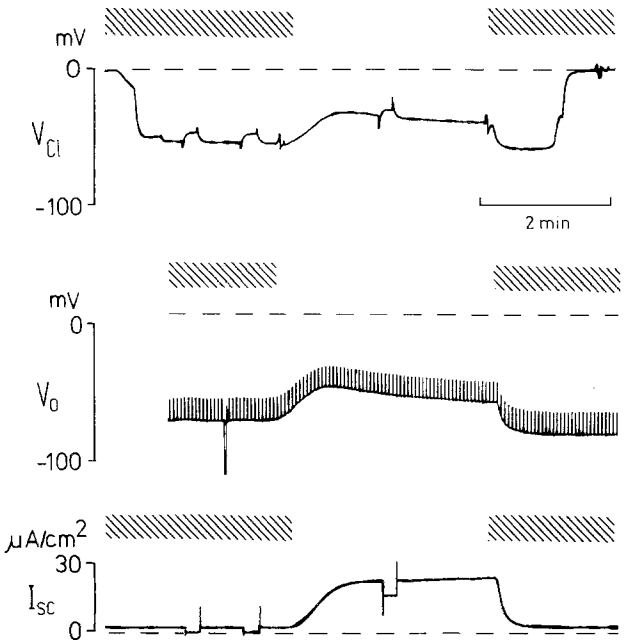


**Fig. 2.** Simultaneous recordings of $V_{Cl}$ and $I_{sc}$ in frog skin epithelium. A recording of $V_o$, taken at a different time in the same experiment, is included for comparison. Hatched bars indicate periods during which amiloride (5 μM) was applied. Transepithelial pulses were not periodically applied in impalements with ion-selective microelectrodes (*see* footnote 2)

From the calibration curve for the $Cl^-$-selective microelectrode used in this experiment, $a^i_{Cl}$ values during the first exposure to amiloride, after 3 min in amiloride-free solution, and during the second exposure to amiloride, were 23.4, 23.4 and 22.9 mM.

Table 1 summarizes the results obtained in experiments with $Cl^-$-selective microelectrodes. It is apparent from this table that $a^i_{Cl}$ did not change significantly following either short-term or long-term exposure to

[2] *F(Ro)* could not be recorded with 150-msec pulses when ion-selective microelectrodes were used. This was because, with the guarded cable used in this study, the response times of these microelectrodes were between 0.5 and 2 sec. As indicated in Figs. 2 and 3, $F(R_o)$ was calculated from the change in microelectrode potential following a 5-sec (for $K^+$ and $Na^+$-selective microelectrodes) or a 10-sec (for $Cl^-$-selective microelectrodes) voltage deflection. These variations in pulse length had no appreciable effect on the value of $F(R_o)$ obtained with open-tip microelectrodes.

**Table 1.** Chloride activity and electrochemical potential difference in short-circuited frog skin[a]

| Experimental conditions | $V_o$ (mv) | $a^i_{Cl}$ (mM) | $a^i_{Cl}/a^{eq}_{Cl}$ | $\Delta\tilde{\mu}_{Cl}/F$ (mV) | $n$ |
|---|---|---|---|---|---|
| Control | $-78\pm9$ | $18\pm3$ | 4.4 | 38 | 5 |
| Amiloride (<5 min) | $-107\pm4$ | $16\pm3$ | 12.3 | 64 | 5 |
| Amiloride (>30 min) | $-90\pm5$ | $20\pm6$ | 7.9 | 53 | 3 |

[a] Effects of amiloride.
Mean values $\pm$ SD are shown for $V_o$ and $a^i_{Cl}/a^{eq}_{Cl}$ and $\Delta\tilde{\mu}_{Cl}/F$ are average values calculated by the Nernst equation from the mean observed values of $V_o$ and $a^i_{Cl}$. $n$ is the number of experiments. Amiloride was added at a final concentration of 2 to 5 μM.

**Table 2.** Potassium activity and electrochemical potential difference in short-circuited frog skin[a]

| Experimental conditions | $V_o$ (mV) | $a^i_K$ (mM) | $\Delta\tilde{\mu}_K/F$ (mV) | $n$ |
|---|---|---|---|---|
| Control | $-68\pm17$ | $132\pm10$ | 40 | 9 |
| Amiloride (<5 min) | $-108\pm5$ | $136\pm18$ | 0 | 9 |
| Amiloride (>30 min) | $-92\pm4$ | $126\pm13$ | 14 | 4 |

[a] Effects of amiloride.
Mean values $\pm$ SD are shown for $V_o$ and $a^i_K$. $n$ is the number of experiments. Amiloride was added at a final concentration of 2 to 5 μM. $\Delta\tilde{\mu}_K/F$ calculated as indicated in Table 1.

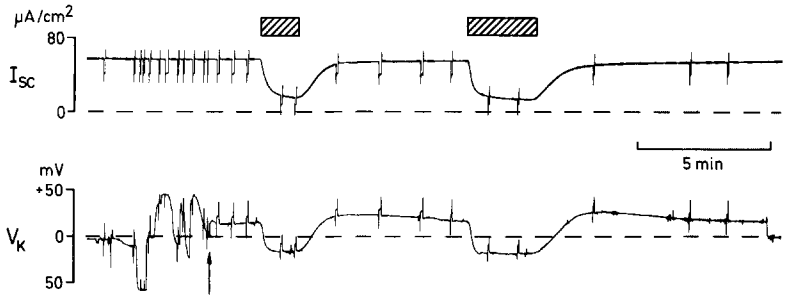


**Fig. 3.** Simultaneous recordings of $I_{sc}$ and $V_K$ in frog skin epithelium. Cell impalement is indicated by arrow. Amiloride (2 μM) was applied during periods indicated by hatched bars. Note that before impalement the imposition of a transepithelial pulse produced only capacitative transients in the $V_K$ tracing. After impalement significant changes in $V_K$ were seen

amiloride. Further, $a^i_{Cl}$ was in all cases clearly above the level corresponding to electrochemical equilibrium across the cell membrane; i.e., there was an outwardly directed (cell-medium) driving force for $Cl^-$. Following prolonged exposure to amiloride, this driving force increased about 1.4-fold compared to the control value, because of the increased electronegativity of the cell interior ($-78$ to $-90$ mV). An even larger increase in the outwardly-directed driving force for $Cl^-$ was observed following short-term exposure to amiloride, but this represents a transient rather than a steady-state situation.

### *Recordings with $K^+$-Selective Microelectrodes*

Figure 3 shows an experiment with a $K^+$-selective microelectrode. In this figure, cell impalement is indicated by the arrow. Before this, and while the microelectrode tip was being advanced through the dense layers of the *stratum corneum*, large and variable potentials were recorded. These potentials clearly occurred before penetration of an epithelial cell membrane since $F(R_o)$ was essentially zero. They were therefore considered artifactual. Following impalement, the microelectrode potential ($V_K$) was +15 mV. The mean value of $V_o$, recorded before and after the events illustrated in Fig. 3, was $-84$ mV. From this and the value of $V_K$ quoted above, $a^i_K$ in this experiment was 139 mM.

Following the first application of amiloride, $V_K$ changed rapidly to $-15$ mV. Combining this with the average value of $V_o$ recorded during this experiment in the presence of amiloride, one obtains $a^i_K = 127$ mM. After 2 min exposure, amiloride was removed from the mucosal medium. When this was done $V_K$ returned initially to a positive value of +23 mV and then declined slowly to +18 mV. Following this, amiloride was again admitted to the mucosal bathing solution and subsequently removed, with a similar sequence of events to those just described.

The results obtained with $K^+$-selective microelectrodes are summarized in Table 2. As was found for $a^i_{Cl}$, $a^i_K$ did not change significantly when the tissue was exposed to amiloride. Under control conditions, $a^i_K$ clearly exceeds the amount corresponding to electrochemical equilibrium (28 mM). Following the application of amiloride, $V_o$ reached a maximal value close to the $K^+$ equilibrium potential ($-108$ mV), but after long term exposure to this agent, $V_o$ declined to a value that was again significantly below this level. Thus, following long-term exposure to amiloride, even though $V_o$ is considerably higher than its

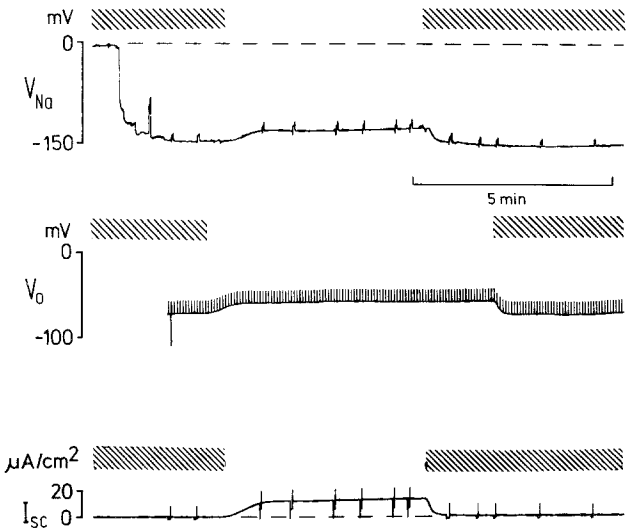


**Fig. 4.** Simultaneous recordings of $I_{sc}$ and $V_{Na}$ in frog skin, together with a recording, during the same experiment, of $V_o$ (*see* Fig. 2). Amiloride (5 μM) was present during periods indicated by hatched bars

value under control conditions, there is a finite outwardly directed driving force for $K^+$.

### *Recordings with $Na^+$-selective Microelectrodes*

Figure 4 illustrates simultaneous tracings of $I_{sc}$ and of $V_{Na}$ measured with a $Na^+$-selective microelectrode. A subsequent measurement of $V_o$ in the same skin is also shown. As already found during $V_{Cl}$ and $V_K$ measurements, $F(R_o)$ values recorded with the $Na^+$-selective and open-tip microelectrodes were essentially the same. Following impalement, which in both cases was done when the tissue had been exposed for more than 80 min to amiloride, $V_o$ was −88 mV and $V_{Na}$ was −146 mV. From these values of $V_o$ and $V_{Na}$ and the calibration curve for the $Na^+$-selective microelectrodes used in this experiment, the calculated value of $a^i_{Na}$ at this time was 6.3 mM. Following removal of amiloride from the apical bathing solution, $V_o$ depolarized from −88 to −74 mV and $V_{Na}$ decreased from −146 to −126 mV. The difference between these changes in potential corresponds to an increase in $a^i_{Na}$ from 6.3 to 8.3 mM during the 5-min period in amiloride-free solution. When amiloride was readmitted to the apical medium, $V_{Na}$ increased to −153 mV and $V_o$ increased to −95 mV. This corresponded to a decrease in $a^i_{Na}$ from 8.3 mM to its initial value in amiloride solution (6.3 mM).

The results obtained with $Na^+$-selective microelectrodes are summarized in Table 3. These show that $a^i_{Na}$ did not change significantly following a short-term exposure of the tissue to amiloride. On the other hand, following a longer exposure to this agent, $a^i_{Na}$ decreased significantly. Following treatment of the tissue with amiloride, the inwardly directed transmembrane driving force for $Na^+$ ($\Delta\tilde{\mu}_{Na}/F$) increased. Thereafter, despite significant changes in $V_o$ and $a^i_{Na}$, $\Delta\tilde{\mu}_{Na}/F$ remained virtually constant.

**Table 3.** Sodium activity and electrochemical potential difference in short-circuited frog skin[a]

| Experimental conditions | $V_o$ (mV) | $a^i_{Na}$ (mM) | $\Delta\tilde{\mu}_{Na}/F$ (mV) | $I_{sc}$ (μA/cm²) | $n$ |
|---|---|---|---|---|---|
| Control | −78±14 | 14±3 | −124 | 30±18 | 4 |
| Amiloride (<5 min) | −108±4 | 14±3 | −154 | 4±2 | 3 |
| Amiloride (>30 min) | −92±4 | 8±3 | −153 | 3±1 | 4 |

[a] Effects of amiloride.
Mean values ±SD shown for $V_o$, $a^i_{Na}$ and $I_{sc}$. $n$ is the number of experiments. Amiloride was added at a final concentration of 2 to 5 μM. $\Delta\tilde{\mu}_{Na}/F$ calculated as indicated in Table 1.

## Discussion

### *Intracellular Potassium Activity*

This study is the first to evaluate intracellular $K^+$ activities in a tight amphibian $Na^+$-transporting epithelium with techniques that allow adequate discrimination between acceptable impalements and those that might be affected by imperfect sealing of the membrane following impalement with a microelectrode, or by other artifacts. Earlier studies on intracellular $K^+$ in frog and toad urinary bladder [6, 16, 17] reported values of $a^i_K$ ranging from 39 to 43 mM. These studies were done by conventional techniques, based on the assumption that the real magnitude of the intracellular potential can be estimated from repeated microelectrode impalements of the tissue. Although this is possible, it has not been verified for the toad urinary bladder, and there is evidence that many reported values of the apical membrane potential are not correct [15]. Since the estimation, with ion-selective microelectrodes, of intracellular ionic activities depends critically upon accurate measurements of membrane potentials and since the membrane potential measurements in these earlier studies are questionable, further studies with techniques similar to those used in the present investigation are desirable. A preliminary study using these techniques [12] gave a mean value of 95 mM for $a^i_K$ in *Necturus* urinary bladder. It is also of interest to note that recent studies which take account of impalement damage have led to a considerable upward revision of the value of $a^i_K$ in toad bladder [5]. On the basis of measurements

with double-barrelled microelectrodes, a value of 81 mM has been proposed for this parameter [7].

We are aware of the problem that microelectrode recordings from the intracellular space of *any* tissue may be affected by artifacts from "pretip potentials" [25]. These offset potentials (some 10–20 mV) were reported to occur in frog skin when low resistance microelectrodes were used and when the microelectrode tip was advanced in an uncontrolled manner into the tissue. Despite the fact that the actual site within the epithelium (*stratum corneum, stratum granulosum, stratum spinosum,* intercellular material) which produces "pretip potentials" has not been identified, we think it unlikely that the present results are influenced by this source of error. Our conclusion rests on the following facts. Microelectrode resistances were always above 20 MΩ, and mechanical deformation of the microelectrode tip (typically seen as an increased microelectrode resistance) occurred only rarely. If it did, it should be noted that offset potentials were observed (Fig. 1). It was, however, often possible (Fig. 1) to reduce the microelectrode resistance to lower values (30–40 MΩ) by drawing back the microelectrode gently for a few μm without damaging the cell. When this was done, the microelectrode potential decreased by a few mV (Fig. 1). In contrast to open-tip microelectrodes, liquid ion-exchanger microelectrodes showed "offset" potentials only before they had passed the dense outer layer of the *stratum corneum* or when they presumably touched the membranes of deeper cell layers (Fig. 3). Otherwise, no indications of artifactual offset potentials, intracellular or extracellular, were found with these microelectrodes. Absence of significant impalement injury was verified repeatedly by addition of amiloride and observation of $V_o$ and $F(R_o)$. In "good" impalements both these parameters consistently increased, $V_o$ to a more negative value and $F(R_o)$ close to 1.0. Similar responses were required for the acceptance of intracellular potentials measured with ion sensitive microelectrodes.

The $a^i_K$ value of 132 mM observed in the present study appears, at first glance, rather high. If one assumes that the activity coefficient for potassium in cytoplasm is essentially the same as in a simple electrolyte solution with the same ionic composition [18], this $a^i_K$ value would correspond to a $K^+$ concentration of some 170 meq/liter cell water. Actual measurements of intracellular $K^+$ concentrations in frog skin by electron microprobe analysis indicate values around 120 meq/kg wet wt [26]. This corresponds to approximately 155 meq K/liter cell water. Taking into consideration that $K^+$-selective microelectrodes are relatively insensitive in this concentration range [11], and allowing for the uncertainties inherent in any estimate of cell water, these values are in reasonable agreement.

Other observations provide evidence in favor of our microelectrode estimate of $a^i_K$. It has been found that, with potassium sulfate Ringer's (110 mM $K^+$) on the serosal side, the potential difference across the basolateral membrane of the frog skin under conditions of zero transcellular current (this is equivalent to the emf of this membrane under conditions where any contribution of a rheogenic or electrogenic pump to the membrane potential may be taken as negligible) is about −10 to −40 mV [22]. With potassium chloride Ringer's, slightly less negative values were observed [8, 24]. By extrapolation from the experimental data in Fig. 6 of Nagel's study [24], it can be estimated that more than 150 mM K ($a^i_K > 125$ mM) in the serosal medium is required for complete depolarization of the basolateral membrane. In addition to supporting our estimated value of $a^i_K$, these data show that contrary to the assumptions of several investigators [10, 20] a complete depolarization of the basolateral membrane cannot be accomplished by incubation with solutions containing 110 mM $K^+$.

It is important to mention that osmotic considerations do not argue against the presence of more than 110 mM $K^+$ in the intracellular space. This is because the anions of the cytoplasm are predominantly of the polyvalent type. As shown below, the chloride concentration of the cell water is around 20 mM and other significant quantities of mono or bivalent anions are most likely absent. Consequently, a high concentration of polyvalent protein anions must be postulated, which does not, however, represent an equivalent osmolality. This is the basis of the well-known Gibbs-Donnan equilibrium, which predicts an excess of mobile cations (and a deficit of mobile anions) for such systems. Our data show that $K^+$ is the major cation that assures the osmotic equilibrium of the frog skin and that $K^+$ has, necessarily, to account for more than 50% of the total intracellular osmolality. These considerations depend strongly on the nature (i.e., the charge density) of the intracellular anions. At present, estimates of this are lacking for frog skin epithelium.

### *Intracellular Chloride Activity*

The mean value of 18 mM observed in this study for $a^i_{Cl}$ in frog skin cells at intracellular potentials averaging −78 mV (Table 1) indicates that $a^i_{Cl}$ in this tight epithelium is 4.4 times the value expected on the basis of passive distribution. From the Nernst equation it is easily calculated that passive equilibrium would exist with an intracellular chloride activity of some 4 mM. Even more evident is the dissociation between

the predicted values on the basis of passive distribution and the observed activities after long term exposure to amiloride. Under these conditions (Table 1), the intracellular potential was hyperpolarized to −90 mV (equivalent to a decrease of the passive distribution concentration of chloride to about 2.5 mM), but the observed values of $a^i_{Cl}$ were virtually unchanged compared to control conditions. This corresponds to an eight-fold accumulation of chloride above the equilibrium level.

Compared with data from electron microprobe analysis, the observed values of $a^i_{Cl}$ appear feasible. Intracellular $Cl^-$ concentration was found to be about 35 meq/kg wet wt [26]. The control $a^i_{Cl}$ value of 18 mM found in this study corresponds to a concentration of about 23 mM in the cell water. The ratio of this value to that reported by Rick et al. [26] is similar to that observed in other epithelia for the relationship between $a^i_{Cl}$ measured with $Cl^-$-selective microelectrodes and the total intracellular $Cl^-$ determined by chemical methods [3, 27]. This may indicate some compartmentation or "binding" of intracellular $Cl^-$ in frog skin. As observed in our present experiments, no change of intracellular $Cl^-$ was found by electron microprobe analysis after treatment with amiloride (A. Dörge, personal communication).

The existence of intracellular $Cl^-$ accumulation in frog skin requires the presence of primary or secondary active steps for $Cl^-$ transport into the intracellular space. The question is, which of the two membranes (apical or basolateral) is responsible for active $Cl^-$ transport? As recently discussed by Zadunaisky [29], frog skins actively transport $Cl^-$ under certain conditions. A small but significant inwardly directed net $Cl^-$ flux has been observed in isolated skin of *R. pipiens* under short-circuit conditions [4]. Also, under open-circuit conditions, Alvarado et al. [2] found that at least 60% of the $Cl^-$ influx is of an active nature. The flux ratio for $Cl^-$ in their study showed a significant discrepancy from the expected value for a purely passive transport mechanism. These results combined with our observation of intracellular $Cl^-$ accumulation indicate that the active step in transcellular $Cl^-$ movement is located at the apical membrane of the epithelial cells[3]. Whether or not $Cl^-$ transport in frog skin, as in some other epithelia [3, 9, 11], is coupled to $Na^+$, is a question that deserves further investigation. From the data of Alvarado et al. [2] and Biber et al. [4] it seems that the coupling between the movement of these ions is very loose, if it exists at all.

[3] It might be argued that $Cl^-$ accumulation in frog skin occurs only in certain specialized cells, e.g., mitochondria-rich cells or gland cells. This, however, would imply the improbable assumption that, in the present experiments, every impalement with a $Cl^-$-selective microelectrode involved one of these cells rather than an epithelial cell, since, in every instance, $a^i_{Cl}$ exceeded its equilibrium value and no evidence was found for heterogeneity among the cells impaled.

*Intracellular Sodium Activity*

The value of $a^i_{Na}$ found in the present study under control conditions is similar to those reported for other epithelia [3, 11]. Furthermore, it agrees well with the value (10.6–16.3 meq/kg wet wt) reported on the basis of electron microprobe analysis for the intracellular $Na^+$ concentration in this tissue [26] and with that obtained from chemical analysis of split skins [1]. The inwardly directed driving force for $Na^+$ ($\Delta\tilde{\mu}_{Na}/F$) calculated from $V_o$ and $a^i_{Na}$ is shown in the last column of Table 3. Under control conditions this had an average value of −124 mV. Under these conditions the mean $I_{sc}$ was 30 μA/cm². Following short-term exposure to amiloride, $I_{sc}$ was reduced to about 10–20% of its control value. At this time $V_o$ had increased to its maximal value (−108 mV) but $a^i_{Na}$ had not changed significantly. Hence, $\Delta\tilde{\mu}_{Na}/F$ had increased to −154 mV. Since this value of $\Delta\tilde{\mu}_{Na}/F$ occurs at a low transepithelial current flow it may be assumed to be close to the maximal driving force ($E_{Na}$) of the $Na^+$ pump. The fact that after long term exposure to amiloride, when $I_{sc}$ had declined to virtually zero and $a^i_{Na}$ has decreased significantly, $\Delta\tilde{\mu}_{Na}/F$ remains unchanged (Table 3) lends support to this idea. Under these conditions the tissue has reached a new steady-state in which net $Na^+$ extrusion *via* the pump has declined to zero or close thereto. Accordingly, the value of about −154 mV may reasonably be assumed to be close to the maximal driving force ($E_{Na}$) of the $Na^+$ pump. The present value is clearly higher than most previous estimates which were derived from transepithelial electrical measurements or flux data (e.g., ref. 14). These, however, may represent a fraction of the actual electrochemical potential of the $Na^+$ pump [28]. In contrast, measurement of the electrochemical potential in the present investigation is directly made across the cell membrane and thus represents a more appropriate estimate of the correct value of $E_{Na}$.

The decline in $a^i_{Na}$ during long-term exposure to amiloride (Table 3) and the changes in $a^i_{Na}$ recorded in the experiment illustrated in Fig. 4, when the tissue was first exposed to amiloride, then to an amiloride-free solution and finally re-exposed to amiloride, strongly supports the existence of a finite $Na^+$ transport-pool in epithelial cells of the isolated frog skin between the $Na^+$ entry and the active $Na^+$ extrusion steps.

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