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Ion transport across the skin of a larval salamander

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The transport characteristics of the skin of neotenic *Ambystoma tigrinum* were investigated using ion substitution and circuit analysis. When bathed with sodium Ringer solution on both sides, a transepithelial potential of up to 50 mV (inside positive) and a short-circuit current (I_{sc}) of up to 10 $\mu A/cm^2$ were observed. When amiloride was added or Na^+ was replaced by tetramethylammonium in the apical solution, I_{sc} was decreased from 3.7 ± 0.4 to 1.5 ± 0.2 $\mu A/cm^2$ ($n = 10$). When K^+ replaced Na^+, there was a smaller change in I_{sc} from 5.8 ± 0.6 to 3.7 ± 0.5 $\mu A/cm^2$ ($n = 10$). Although barium had no effect when added to 100 K Ringer on normal skin, it inhibited I_{sc} on skins taken from K^+-loaded animals. Nystatin caused substantial increases in I_{sc} with either Na^+ or K^+ as the dominant cation in the apical solution. Current voltage analysis using amiloride was used to estimate the resistances and electromotive forces (EMF) associated with ion transport. The EMF for ion transport was partially dependent on K^+ in the basolateral solution and it was similar to that observed in other epithelia. The resistance of the transport pathway was high, consistent with the low I_{sc}. These results suggest that there is an amiloride-sensitive Na^+ channel in parallel with a small K^+ conductance in the apical membrane of this preparation.

Introduction

Larval amphibians, being obligately aquatic, face quite different osmotic and ionic regulation problems than the semi-terrestrial adults. There are the obvious problems of water loading and ion loss that can apparently be largely compensated for by feeding and renal excretion [1]. The role of the skin in this overall ionic balance is not as clear. In adults, there is a relatively large amount of sodium transported from the apical to the basolateral side [2]. In larvae, transport rates are generally much lower [1–9], although they have the capacity for high rates of transport if Na^+ is allowed to enter the cell [4,10,11]. The apical membrane of the larval skin appears to have fewer transport proteins than the adult, these may be relatively non-selective for the alkali metal cations [7,11]. In addition, amiloride stimulates ion transport in larval frog skin [8,12] instead of the marked inhibition observed in most other epithelial Na^+ transport systems [13].

The purpose of the present study was to examine carefully the ion transport properties of the larval salamander skin. It has previously been shown that a significant transepithelial potential develops across larval salamander skin [1] that is dependent on external Na^+ [6]. In addition, amiloride has been shown to partially inhibit the short-circuit current [2]. The present study confirms and extends these observations. With circuit analysis using amiloride I will show that the Thevenin equivalent electromotive force (EMF) for Na^+ transport in larval salamander skin is comparable to that in other epithelia. The low short-circuit current is due to a high-resistance

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active pathway. In addition, there is some evidence to suggest the presence of a K^+ channel in parallel to the Na^+ transport system.

Methods

Neotenous tiger salamanders (*A. tigrinum*) were purchased from Charles D. Sullivan, Nashville, TN, and kept in refrigerated tap water. A special group of salamanders were kept in refrigerated tap water to which 15 mM KCl had been added (see results). They were not fed and were used within 1–2 weeks of shipment. Animals were anesthetized in 0.1% tricaine methanesulfonate (Sigma Chemical Co., St. Louis, MO) with the pH adjusted to 7.0 with $NaHCO_3$.

The skin is tightly attached to the underlying musculature on most parts of the body except between the two forelegs on the ventral side, where pieces as large as 1.5 cm^2 may be removed with minimal damage. Isolated skins were placed in special chambers (0.72 cm^2) that permitted the tissues to be short-circuited continuously during solution changes. Silicone grease (Dow Corning, Midland, MI) formed a tight seal between skin and chambers [5].

Transepithelial potential and short circuit current were measured with 1 M NaCl or 3 M KCl salt bridges connected to the voltage clamp via Ag|AgCl wires [5,11,15]. NaCl salt bridges were used unless otherwise noted. Previous studies of other epithelia have shown that studies of current voltage relationships can yield information on the electromotive force for ion transport and resistances of the active and shunt pathways [14–16]. Current-voltage (*I-V*) plots were generated by clamping transepithelial potential to a prescribed voltage for 600 ms and measuring the change in clamp current (I_T). The last 50 ms of the pulse was sampled, averaged, and stored on disc using a laboratory microcomputer (Hewlett-Packard, Model 9825A). The data were later recalled and plotted for analysis of the current voltage relationship. Transepithelial potential and I_T were also displayed on a fast strip chart recorder (Gould Brush Model 220). During most pulses, transepithelial potential and I_T were in a 'steady state' during the interval sampled by the computer (see Results).

Sodium Ringer (100 Na) solution contained, in mM: 100 NaCl, 2.0 CaCl, 2.4 $KHCO_3$ (pH 8.2). Potassium Ringer (100 K) and tetramethylammonium Ringer were identical to 100 Na except that 100 K and 100 tetramethylammonium were substituted for Na^+, respectively. 24 K Ringer was made by simple replacement of K^+ for Na^+. Amiloride (Merck, Sharp & Dohme Research Laboratories, West Point, PA) was used at a final concentration of 0.1 mM. Nystatin (Sigma Chemical Co., St. Louis, MO) was dissolved in methanol (12000 U/ml) and then added to the Ringer solution. Barium chloride was added directly to the appropriate Ringer solution.

Results

Sodium dependence

When bathed by 100 Na Ringer solution on both sides, the larval salamander skin develops a substantial transepithelial potential of up to 50 mV (inside positive). When transepithelial potential was clamped to zero, a short-circuit current of up to 10 $\mu A/cm^2$ was observed. Transepithelial resistance (R_T) ranged from 2200 to 16000 $\Omega \cdot cm^2$.

I_{sc} was dependent on Na^+ in the apical solution. When 100 Na Ringer was replaced with tetramethylammonium Ringer, I_{sc} rapidly decreased from 7.3 to 2.8 $\mu A/cm^2$ (Fig. 1). Re-

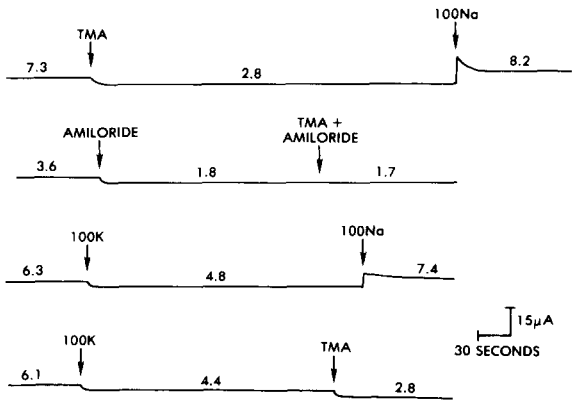

Fig. 1. Response of I_{sc} to ion substitution and amiloride in the apical solution. Numbers indicate absolute values for I_{sc}. TMA is tetramethylammonium chloride Ringer. Other abbreviations are defined in the text.

peated flushing with the Na^+-free Ringer caused no further decrease in I_{sc}. There was also only a very small change in I_{sc} over 2–3 min incubation with the same solution, suggesting that Na^+ diffusing from the salt bridges was not a major contributor to the residual I_{sc}. Measurement of Na^+ concentration in the apical solution (tetramethylammonium Ringer) indicated that about 0.2 mM remained. Two experiments were performed using 3 M KCl salt bridges to avoid Na^+ contamination from the salt bridges. Similar results to those using 1 M NaCl salt bridges were obtained. In either case, when 100 Na Ringer was returned, there was an immediate rise in I_{sc} that decayed rapidly to values slightly above control.

The Na^+-dependent portion of the I_{sc} was blocked by amiloride. Amiloride (0.1 mM) caused I_{sc} to decrease from 3.6 to 1.8 $\mu A/cm^2$ (Fig. 1). Removal of Na^+ had no additional effect. In ten skins, amiloride reduced I_{sc} from 3.7 ± 0.4 to 1.5 ± 0.2 $\mu A/cm^2$. This is a markedly different response than that observed in tadpole skin, where I_{sc} was often transiently stimulated by amiloride [5,8,12].

A more quantitative characterization of the transport properties of the skin can be obtained from circuit analysis. The Thevenin equivalent driving force for transepithelial ion transport (E_A) is considered in its original context [17]. This model also contains an active pathway resistance (R_A) and a parallel shunt resistance (R_{sh}). Once E_A is known, it is a simple matter to calculate the resistance of the active and shunt pathways. The low transport rate observed in salamander skin could be due to a poorly developed Na^+ extrusion mechanism at the basolateral membrane or a high resistance entry step at the apical membrane. Direct estimates of E_A and R_A in combination with the nystatin data should help resolve this question.

The circuit was probed using *I-V* analysis in combination with amiloride. In most cases, when transepithelial potential was clamped to the new value, the clamp current went through a small transient and then stabilized after 150–200 ms. This 'steady state' value was averaged as described in Methods and taken as the current at that voltage. In a few cases, clamp current started to rise after the initial transient and continued to increase. This generally occurred at voltages higher than the 'inflection' point and was much more frequently observed with 24 K Ringer in the basolateral solution (see below).

Amiloride has been used in many epithelia to selectively increase R_A [13,18,19]. It is theoretically possible to generate a family of lines on an *I-V* plot with a common point which corresponds to E_A. In this analysis it is assumed that amiloride does not affect E_A or R_{sh}. This is generally thought to be true in most epithelia, although there is some evidence for small effects on E_A and R_{sh} [13, 18–20].

The effect of amiloride on the *I-V* relationship is shown in Fig. 2. As shown here (solid line), the *I-V* relationship was linear up to about 130 mV. A straight line was drawn by linear regression of the lower seven points. Above 130 mV, the points showed a significant deviation from the projected straight line. After amiloride (0.1 mM) treatment, a second *I-V* relationship was obtained. There was a significant decrease in I_{sc} and an increase in the slope (R_T). Again, a straight line was drawn

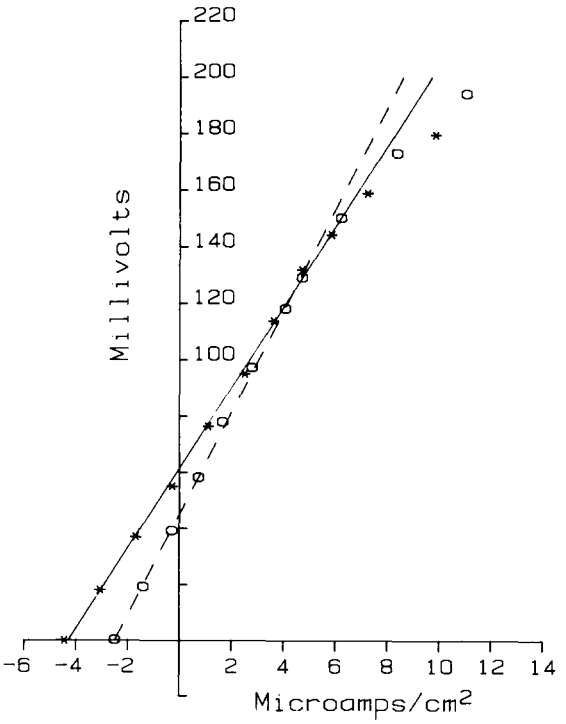

Fig. 2. Representative *I-V* relationships for larval salamander skin. Data were generated under control conditions (stars) and after 0.1 mM amiloride treatment (open circles). Lines were drawn using linear regression (see text for details).

through the first seven points (dashed line). As in control conditions, there was significant deviation of the data points from the projected line at voltages greater than 130 mV.

If the above assumptions about amiloride effects on E_A and R_{sh} are valid, amiloride should cause the *I-V* plot to rotate about E_A; the intersection of the two straight lines of Fig. 2 should occur at the point corresponding to E_A on the ordinate. E_A calculated in this way was 122 mV and R_{sh} was 29 $k\Omega \cdot cm^2$. In ten similar experiments the mean values for E_A and R_{sh} were 126 ± 8.8 mV and 14.7 ± 2.3 $k\Omega \cdot cm^2$. This value for E_A is similar to that observed in many other epithelia [14,15,17–19,23].

A second approach was taken to estimate E_A in these studies. As noted above, at points above 120–130 mV, the *I-V* relationship deviates significantly from the projected straight line. This rectification has been shown to occur in many epithelia and the inflection point is thought to coincide with other estimates of E_A [14–16,18,19,23]. The inflection point was determined statistically by assigning data points to regions above and below the point where deviations from linearity were observed. Pairs of regression lines were drawn by incrementing the number of points assigned to the upper and lower regions. The pooled residual sum of squares was calculated for the various regression line combinations. The best estimate of the true inflection point was calculated from the intersection of the two regression lines that gave the minimum pooled residual sum of squares [21]. The values for E_A and R_{sh} determined in this way for Fig. 2 were 140 mV and 25.0 $k\Omega \cdot cm^2$. The average 'inflection point' values for the same ten skins that were treated with amiloride were 132 ± 4.0 mV and 14.3 ± 2.2 $k\Omega \cdot cm^2$. These values are not significantly different from those determined from the amiloride method.

Nystatin had rapid and large effects on I_{sc} and R_T. With 100 Na outside, nystatin (120 U/ml) increased I_{sc} from 5.3 ± 0.3 to 19.7 ± 3.5 $\mu A/cm^2$ and decreased R_T from 4620 ± 653 to 2086 ± 350 $\Omega \cdot cm^2$ ($n = 3$). Methanol alone at the concentrations used above had no effect on the skin.

Potassium dependence

In frog tadpole skin, the apical membrane has a significant permeability to K^+ in addition to Na^+ [5,7]. The following experiments were done to check for a K^+ permeability in salamander skin. When 100 Na Ringer was replaced by 100 K Ringer, there was a small but significant decrease in I_{sc} (Fig. 1). No further decrease in I_{sc} was observed during repeated flushing with 100 K Ringer. When 100 Na Ringer was returned there was an immediate rise in I_{sc} that decayed to values approaching control over several minutes (Fig. 1). In ten experiments, substitution of 100 Na Ringer with 100 K Ringer reduced I_{sc} from 5.8 ± 0.6 to 3.7 ± 0.5 $\mu A/cm^2$. These numbers are significantly different using a paired *t*-test ($P < 0.05$). Two of the above experiments were done using 3 M KCl salt bridges. The results were not different from those obtained using NaCl salt bridges. It should be noted that in one skin there was virtually no change in I_{sc} and in two skins there were large changes comparable to that observed with Na^+ removal. There were corresponding R_T changes suggesting that the change in I_{sc} was due to effects on the epitheLia and not due to bridge junction potentials. Neither amiloride (not shown) nor 5 mM barium (Fig. 3) could be shown to affect I_{sc} with 100 K Ringer outside in these skins. The lack of an amiloride effect suggests that the residual current is not due to any remaining low levels of Na^+, at least not Na^+ movement through amiloride-sensitive Na^+ channels.

Replacement of Na^+ by K^+ caused a significantly smaller ($P < 0.05$) average change in I_{sc} than that observed when amiloride was added to 100 Na Ringer. Amiloride inhibited I_{sc} to $41.4 \pm 5.0\%$ (10) of control in 100 Na Ringer, while K^+ replacement reduced I_{sc} to only $64.5 \pm 7.0\%$ (10)

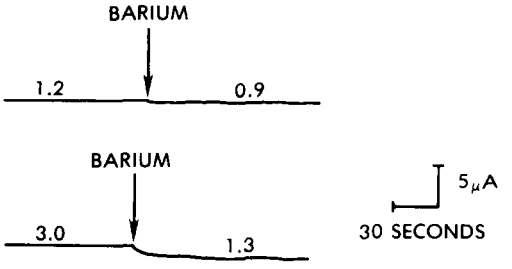

Fig. 3. Response of I_{sc} to 5 mM barium in the apical solution (100 K Ringer). Top trace (normal animal); bottom trace (K^+-loaded animal). See text for details.

of control. When 100 K Ringer was replaced with tetramethylammonium Ringer (Fig. 1), I_{sc} dropped from 4.4 to 2.8 $\mu A/cm^2$. These results suggest that there may be a small K^+ conductance at the apical membrane.

Recent experiments on frog skin have shown that keeping the animals in 15 mM KCl for several days causes the induction of K^+ channels in the apical membrane [22]. A similar protocol was tried on the larval salamander. Salamanders were kept in tap water with 15 mM KCl for 6–8 days. The animals were then killed and the skin was examined in the usual way.

With 100 Na Ringer on both sides, I_{sc} was larger than previously observed in 'normal' salamanders, although the ranges overlapped. I_{sc} and R_T were $7.0 \pm 1.9\ \mu A/cm^2$ and $10.4 \pm 2.3\ k\Omega \cdot cm^2$ ($n = 8$), respectively. In three experiments, amiloride was added to 100 Na^+ in the apical solution. The response was essentially identical to that observed in 'normal' skins. When 100 K Ringer replaced 100 Na Ringer on the apical side, I_{sc} and R_T were changed to $2.9 \pm 0.7\ \mu A/cm^2$ and $11.6 \pm 2.0\ \Omega \cdot cm^2$, respectively. These results are not different from those obtained on the 'normal' salamanders. However, when 5 mM BaCl was added to the apical solution, there was often a significant decrease in I_{sc}. The largest effect was a reduction from 3.0 to 1.3 $\mu A/cm^2$ (see Fig. 3). One skin in this group did not respond to barium. In seven skins, barium reduced the I_{sc} to $68 \pm 6\%$ of the value in 100 K Ringer. This effect was significant ($P < 0.05$, paired t-test).

Nystatin had rapid and large effects on I_{sc} and R_T in 'normal' salamanders when applied with 100 K Ringer in the apical solution. Nystatin (120 U/ml) increased I_{sc} from 4.0 ± 0.6 to 20.7 ± 3.8 $\mu A/cm^2$ and decreased R_T from 5107 ± 784 to $1727 \pm 334\ \Omega \cdot cm^2$ ($n = 3$).

A set of experiments was done where K^+ was increased symmetrically. It was anticipated that E_A would be dependent on K^+ concentration in the basolateral solution [15,23]. Symmetrical solutions should eliminate uncertainties due to diffusion potentials developing at the salt bridges or across the shunt. Amiloride was also used to evaluate possible changes in E_A and R_{sh} due to increased K^+ concentration.

When 24 K Ringer was placed on both sides of

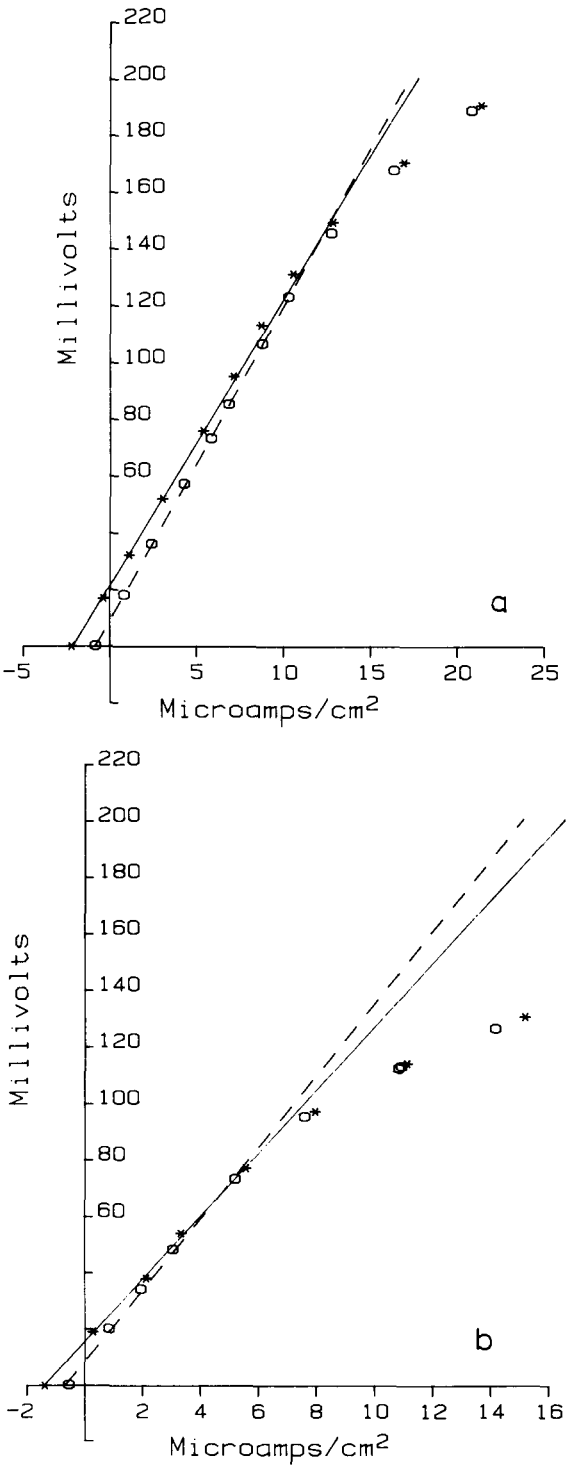

Fig. 4. (a) Representative I-V relationships under control (stars) and after amiloride treatment (open circles). 100 Na Ringer both sides. (b) Same skin as (a) only with 24 K Ringer on both sides.

the tissue, I_{sc} decreased from 3.5 ± 0.8 to 2.5 ± 0.6 [4] $\mu A/cm^2$. R_T declined slightly from 9.3 ± 0.7 to 8.6 ± 0.2 $k\Omega \cdot cm^2$. The drop in I_{sc} with no increase in R_T suggested that E_A had declined. It could also have indicated an increase in R_A with a compensating decrease in R_{sh}. Fig. 4a shows an experiment similar to that of Fig. 2. Clamp current reached steady-state values during the clamp interval. E_A calculated from the intersection of control and amiloride lines was 147 mV compared to E_A of 143 mV determined from the inflection point. R_{sh} was 11.8 and 12.5 $k\Omega \cdot cm^2$ determined by the two methods, respectively. Fig. 4b shows an experiment performed on the same skin with 24 K Ringer on both sides. In these experiments clamp current did not reach a steady state during the usual time interval at all voltages examined. In most of these experiments at voltages above 60–70 mV, the clamp current went through the usual initial transient and then started to increase over the next several hundred milliseconds. At voltages below this, clamp current reached a steady state and the first five points were used to calculate the straight lines. In this experiment, E_A determined from the amiloride technique was 74 mV and R_{sh} was 13.5 $k\Omega \cdot mc^2$. Inflection point analysis was not performed in these experiments. In four experiments, E_A declined from 124 ± 9.2 to 93 ± 7.1 as determined from intersecting lines. R_{sh} went from $12.5 \pm$ to 12.1 ± 0.6 $k\Omega \cdot cm^2$. With essentially no change in R_T or R_{sh} and hence no change in R_A, the drop in I_{sc} upon the introduction of 24 K Ringer was apparently not due to an increase in resistance. The decline appears to be due almost exclusively to a decrease in E_A. In fact, the average decline in I_{sc} was to 71% of control and the average drop in E_A was to 75% of control. The classic model for Na^+ transport predicts that E_A will be dependent on K^+ concentration in the basolateral solution [23].

Discussion

While it has previously been shown that there is a low rate of amiloride-sensitive Na^+ transport across the skin of the larval salamander [2], few details of the transport mechanisms have yet been established. The purpose of the present study was to characterize the transport mechanisms of Na^+ and K^+ across the salamander skin. In the 'normal' salamander skin, Na^+ is transported at a low rate through amiloride-sensitive channels. Circuit analysis revealed a very high resistance pathway for Na^+ transport in series with an EMF dependent on basolateral K^+ concentration similar to that observed in other epithelia. There was evidence for a small apical membrane K^+ conductance in these animals. When compared, the ion transport properties of the larval salamander skin appear to resemble more closely those of the adult frog skin rather than its larval counterpart.

E_A (amiloride method) was found to be in the range of 100–150 mV, a value very similar to that observed in other epithelia [14–19,24]. Calculation of active pathway resistance showed it to be large and primarily responsible for the low I_{sc}. The results of the nystatin experiments provide strong support for this conclusion. In addition, E_A was shown to be partially dependent on K^+ in the basolateral solution. This is to be expected if E_A can be modeled as a Thevenin equivalent of a pump and a K^+ channel in parallel at the basolateral membrane [15].

A second approach to circuit analysis was also used. This more speculative method assumes that current flow through the active pathway rectifies. Thus, when transepithelial potential equals E_A, current flow through the active pathway changes direction. This would be reflected as a change in transepithelial resistance if the transport pathway rectifies. It was observed by Civan [24] and later more completely developed by Helman and colleages [14,15] that distinct breaks or 'inflection points' occur at voltages that coincide directly with the Thevenin EMF for transport (E_A) determined by other techniques. In the present study, E_A determined from 'inflection point' analysis was shown to be identical to (or perhaps coincidental with) E_A determined using the amiloride technique.

The Na^+ dependence of I_{sc} was examined. Even after a nominally complete removal of Na^+, there was still a significant I_{sc}. This persisted even when amiloride was added to the tetramethylammonium Ringer and when 3 M KCl bridges were used to minimize the leakage of Na^+ from the salt bridges. If this residual I_{sc} is due to Na^+ transport, it would have to be through a very high-af-

finity, amiloride-insensitive channel. Since there was a small amount of K^+ around in these experiments (2.4 mM), I considered the possibility that there might be a significant K^+ pathway.

When K^+ replaced Na^+, there was a significant decline in I_{sc}. It initially appeared that this was essentially a response identical to that obtained when amiloride was added to 100 Na Ringer, only to a lesser degree. Since we are dealing with an asymmetrical system the interpretation of the results may be complicated by junction potentials developing at the salt bridges [25] and by diffusional EMFs developing across the shunt pathway. Similar results were obtained using either 1 M NaCl or 3 M KCl salt bridges, suggesting that bridge junction potentials were not a major problem. To eliminate diffusional EMFs across the shunt, it may be necessary to do to a symmetrical substitution of K^+ for Na^+. However, K^+ may depolarize the basolateral membrane markedly changing the transport characteristics of the epithelium. The results of this experiment would not necessarily provide any new information on the permeability characteristics of the apical membrane. In fact, when K^+ was raised to 24 mM symmetrically, I_{sc} was decreased primarily by a decrease in E_A. Another approach is to use specific inhibitors in the apical solution. Barium has been shown to inhibit K^+ channels in the apical membrane of amphibian epithelia [22,26]. When barium was added to 'normal' skins, there was no effect. However, after K^+ loading of the animals, a small inhibition of I_{sc} by barium was observed. Although it could be argued that this was due to barium effects on the shunt pathway, it is more strongly suggestive of a small K^+ conductance as observed in other systems [22].

Physiological implications

The apical membrane of the salamander skin possesses an amiloride-inhibitable Na^+ conductance with possibly a small, parallel K^+ conductance. After K^+ loading of the animals, barium sensitivity is evident. From what we know about the basolateral membrane, it appears to be similar to that of other Na^+-transporting epithelia. The EMF for ion transport is in the same range as that of other epithelia and is dependent on K^+ in the basolateral solution. Although the salamander skin transports Na^+ at a lower rate than the adult frog skin, its transport properties are very similar to the frog. This is surprising in view of the fact that it would seem to face the same ionic and osmotic stresses as the frog tadpole. Yet the frog tadpole skin responds quite differently to amiloride and appears to have a nonselective channel rather than two channels in parallel. These differences might be related to the relative age of these animals. The studies reported here were performed on neotenous larval forms. Previous studies on non-neotenic animals have shown that the skin is unresponsive to amiloride and has an apparently larger K^+ conductance at the apical membrane than that observed in the present studies (unpublished observations). These responses are more like those observed in the frog tadpole skin.

It is not clear at the present time what adaptive role the tight apical membrane may play in the overall ionic balance of the animal. It may serve simply to prevent salt loss to the pond, with ion regulation being accomplished primarily by the gills. However, the response to K^+ loading suggests that the skin may have a role in K^+ balance [22,27]. The enhancement of a K^+ channel could enable the skin to secrete K^+ should the need arise.

It is clear from circuit analysis and the nystatin studies that the skin has the potential for high rates of ion transport. It may be that with the resorption of the gills at metamorphosis a new ion regulatory site is needed. A high transport capacity could be rapidly established by simply inserting or modifying transport proteins in the apical membrane.

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