Possible role of aldosterone and T\textsubscript{3} in development of amiloride-blockable SCC across frog skin in vivo

MAKOTO TAKADA, MICHIO SHIIBASHI, AND MIYOKO KASAI
Department of Physiology, Saitama Medical School, Moroyama, Iruma-gun, Saitama 350–0495, Japan

Takada, Makoto, Michio Shiibashi, and Miyoko Kasai. Possible role of aldosterone and T\textsubscript{3} in development of amiloride-blockable SCC across frog skin in vivo. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R1305–R1312, 1999.—There are inconsistencies between the in vivo and in vitro effects of thyroid hormone and aldosterone (Aldo) on the development of an amiloride-blockable short-circuit current (SCC) across bullfrog skin [Takada, M., H. Yai, and K. Takayama-Arita. Am. J. Physiol. 268 (Cell Physiol. 37): C218–C226, 1995]. To address this issue, tadpoles were raised in the presence of Aldo and T\textsubscript{3}. An amiloride-blockable SCC developed across the skin before forelimbs appeared. Noise analysis of the characteristics (single-channel current, blocking and unblocking rate coefficients, and apparent dissociation constant) of this amiloride-blockable Na\textsuperscript{+} channel showed that it really was of the adult type. A similar SCC developed at stage XIX in the skin of tadpoles raised with Aldo alone. These results strongly support our hypothesis that the crucial hormone in the development of this SCC is Aldo but that a suppression mechanism attenuates its effect on SCC development until it is removed by the increase in the serum concentration of thyroid hormone (which starts at stages XVIII–XIX in vivo).

amphibian metamorphosis; epithelial remodeling; cell differentiation; noise analysis; active Na\textsuperscript{+} transport

ACTIVE Na\textsuperscript{+} TRANSPORT, measured as the amiloride-blockable short-circuit current (SCC) across bullfrog skin, develops during the climax stages of metamorphosis (TK stages XXI–XXII); i.e., it becomes detectable after the appearance of the forelimbs at stage XX, and it increases thereafter (3, 7, 22, 30). The development of this transport has been assumed to be induced by thyroid hormone, aldosterone (Aldo), hydrocortisone, or corticosterone, because the serum concentration of these hormones increases during metamorphosis (12, 14, 16, 18, 21). However, exactly which hormone(s) are responsible for its development is a controversial issue (6, 23). For example, not only the forelimbs but also the transport developed when tadpoles at stages XIII–XV were raised in the presence of thyroid hormone for 2 wk, but neither the forelimbs nor the transport developed when similar tadpoles were raised with Aldo, hydrocortisone, or corticosterone for the same period (23). In contrast, in in vitro studies thyroid hormone did not induce the development of this current, but corticoids, especially Aldo, did induce its development when EDTA-treated isolated tadpole skin was cultured with these hormones (24, 27).

Thus the reported effects of thyroid hormone and Aldo on the development of the transport are inconsistent between the in vivo and in vitro situations. We hypothesized that Aldo is crucial for the development of this current, but that its effect is suppressed by some means until the suppression is removed by the increase in the level of endogenous thyroid hormone that occurs before stage XX in vivo. If this is correct, the reported effects of Aldo and thyroid hormone in vivo and in vitro no longer seem inconsistent. In actual fact, the serum concentration of thyroid hormone starts to increase at stages XVIII–XIX (18, 21).

If the above hypothesis is correct, the transport should develop before stage XXI when tadpoles are raised in the presence of Aldo and T\textsubscript{3}. In this study, we set out to see if this does indeed occur.

MATERIALS AND METHODS

Animals and dissection of dorsal skin. Tadpoles of Rana catesbeiana at stages X–XIII (purchased from a local animal supplier in Misato City, Saitama, Japan) were maintained in tap water or in 10\textsuperscript{–6} M Aldo, 10\textsuperscript{–8} M T\textsubscript{3}, or 10\textsuperscript{–6} M Aldo + 10\textsuperscript{–8} M T\textsubscript{3} (Aldo + T\textsubscript{3}) for 5–11 days. The stages were determined by reference to the work of Taylor and Kollros (29). Both hormone-treated and hormone-untreated larvae were anesthetized with iced water supplemented with MS-222, and portions of dorsal body skin were dissected out.

Culture of dorsal skin of tadpoles. Two kinds of skin at stages XI–XV were used: 1) EDTA-treated skin and 2) non-EDTA-treated skin. To produce EDTA-treated skin, the dissected skin was washed with 70% ethanol and then with 2.5 mM EDTA to remove larval-type cells, such as apical and skin cells, and then transferred to tissue culture medium as described previously (27). To produce non-EDTA-treated skin, the dissected skin was cultured directly with culture medium without treatment by ethanol and EDTA. RPMI-1640 (GIBCO, Grand Island, NY) was diluted to 70% with distilled water, supplemented with 10\textsuperscript{–6} M Aldo, 16.7 mM NaHCO\textsubscript{3}, 10 mM HEPES (pH 7.4), 100 IU/ml penicillin, and 100 µg/ml streptomycin, and used as the culture medium. Each type of skin was cultured in a humidified atmosphere of 5% CO\textsubscript{2} and 95% room air at 24°C for 2 wk.

Light microscopy and immunocytochemistry. Dorsal skin from tadpoles raised with Aldo and T\textsubscript{3} for 11 days and non-EDTA-treated tadpole skin that had been cultured with Aldo for 2 wk were each fixed with 10% paraformaldehyde and embedded in paraffin. Sections (8 µm) were stained with hematoxylin and eosin and viewed under a light microscope. Sections for immunocytochemistry were prepared as described above, and the localization of human blood group antigen A was detected by means of a standard method, as described previously (27).
Electrical measurements

Dissected skin or cultured skin samples were mounted in a Ussing-type chamber with silicone gaskets (ID 5 mm) to minimize edge damage. Both sides of the skin samples were bathed in aerated Ringer solution containing (in mM) 110 NaCl, 2 KCl, 1 CaCl₂, 10 glucose, and 10 Tris at pH 7.2. The transepithelial potential difference across the skin, SCC, and skin resistance (R) were measured as previously described (27).

The method used for current fluctuation (noise) analysis was as follows. To produce a low-noise device suitable for our present purposes, an SCC amplifier (CEZ-9100) was specially modified by M. Makimoto (Nihon Kohden, Tokyo). The fluctuations in SCC were high-pass filtered (0.05 Hz), amplified \( \times 500 \), and low-pass filtered (250 Hz) to prevent aliasing errors. The signal was sampled at 512 Hz (i.e., every 1.95 ms) for 8 s (giving a record of 4,096 points). Then a power density spectrum (PDS) was calculated for each of these records with a digital spectrum analyzer (R-9211 A; Advantest, Tokyo). Twenty such PDSs were collected from sequential records at a given amiloride concentration, and from these an average PDS was calculated for each concentration of the drug. The spectra were represented in a double-logarithmic plot (power vs. frequency). Analysis of the PDS yields the Lorentzian parameters \( S_0 \) (plateau) and \( F_c \) (corner frequency) as follows

\[
S(F) = S_0[1 + (F/F_c)^2] + S_{11}/F
\]

where \( S_{11} \) is the power of fluctuations of frequency \( F \), \( S_0 \) is the power of the \( 1/F \) component at 1 Hz, and \( \alpha \) is the exponent that defines the slope of the \( 1/F \) component (4, 5, 7, 17). The \( \text{Na}^+ \) channel block by amiloride is assumed to be described by a two-state model of open-block channel kinetics

\[
2\pi F_c = K_{01} \cdot [A] + K_{10}
\]

where \( K_{01} \) and \( K_{10} \) are the blocking and unblocking rate coefficients, respectively, and \( [A] \) is the concentration of the blocker, amiloride (17). The single-channel current (i) and the \( \text{Na}^+ \) channel density (M; sum of open and blocked channels) were calculated from

\[
i = S_0(2\pi F_c)^2/4 \cdot I_{\text{Na}} \cdot K_{01} \cdot [A]
\]

and

\[
M = (I_{\text{Na}} - 2\pi F_c)/(i \cdot K_{10})
\]

in which \( I_{\text{Na}} \) is calculated by subtracting the amiloride-insensitive SCC from the total SCC at each amiloride concentration (17). The values for \( i \) and \( M \) given in the RESULTS section were determined from the means of the values obtained at each amiloride concentration (1, 2, 6, and 12 µM). The maximum effect of amiloride on the SCC was achieved at 36 or 100 µM, and the remaining SCC was assumed to be amiloride-insensitive. \( I_{\text{Na}(\text{amil})} \) was calculated by subtracting the amiloride-insensitive SCC from the total SCC under amiloride-free conditions.

Electrical measurements

Dissected skin or cultured skin samples were mounted in a Ussing-type chamber with silicone gaskets (ID 5 mm) to minimize edge damage. Both sides of the skin samples were bathed in aerated Ringer solution containing (in mM) 110 NaCl, 2 KCl, 1 CaCl₂, 10 glucose, and 10 Tris at pH 7.2. The transepithelial potential difference across the skin, SCC, and skin resistance (R) were measured as previously described (27).

The method used for current fluctuation (noise) analysis was as follows. To produce a low-noise device suitable for our present purposes, an SCC amplifier (CEZ-9100) was specially modified by M. Makimoto (Nihon Kohden, Tokyo). The fluctuations in SCC were high-pass filtered (0.05 Hz), amplified \( \times 500 \), and low-pass filtered (250 Hz) to prevent aliasing errors. The signal was sampled at 512 Hz (i.e., every 1.95 ms) for 8 s (giving a record of 4,096 points). Then a power density spectrum (PDS) was calculated for each of these records with a digital spectrum analyzer (R-9211 A; Advantest, Tokyo). Twenty such PDSs were collected from sequential records at a given amiloride concentration, and from these an average PDS was calculated for each concentration of the drug. The spectra were represented in a double-logarithmic plot (power vs. frequency). Analysis of the PDS yields the Lorentzian parameters \( S_0 \) (plateau) and \( F_c \) (corner frequency) as follows

\[
S(F) = S_0[1 + (F/F_c)^2] + S_{11}/F
\]

where \( S_{11} \) is the power of fluctuations of frequency \( F \), \( S_0 \) is the power of the \( 1/F \) component at 1 Hz, and \( \alpha \) is the exponent that defines the slope of the \( 1/F \) component (4, 5, 7, 17). The \( \text{Na}^+ \) channel block by amiloride is assumed to be described by a two-state model of open-block channel kinetics

\[
2\pi F_c = K_{01} \cdot [A] + K_{10}
\]

where \( K_{01} \) and \( K_{10} \) are the blocking and unblocking rate coefficients, respectively, and \( [A] \) is the concentration of the blocker, amiloride (17). The single-channel current (i) and the \( \text{Na}^+ \) channel density (M; sum of open and blocked channels) were calculated from

\[
i = S_0(2\pi F_c)^2/4 \cdot I_{\text{Na}} \cdot K_{01} \cdot [A]
\]

and

\[
M = (I_{\text{Na}} - 2\pi F_c)/(i \cdot K_{10})
\]

in which \( I_{\text{Na}} \) is calculated by subtracting the amiloride-insensitive SCC from the total SCC at each amiloride concentration (17). The values for \( i \) and \( M \) given in the RESULTS section were determined from the means of the values obtained at each amiloride concentration (1, 2, 6, and 12 µM). The maximum effect of amiloride on the SCC was achieved at 36 or 100 µM, and the remaining SCC was assumed to be amiloride-insensitive. \( I_{\text{Na}(\text{amil})} \) was calculated by subtracting the amiloride-insensitive SCC from the total SCC under amiloride-free conditions.
Statistical analyses. Statistical significance was assessed with a one-way ANOVA followed by Scheffé’s test (for three groups) or by Student’s t-test or Welch’s test (for two groups).

RESULTS

Acetylcholine and amiloride responses in skin from naturally metamorphosed tadpoles. The ACh or amiloride effect on the SCC is used as a functional marker to distinguish whether bullfrog skin is larval type or adult type, because 1) apical application of ACh (1 mM) or amiloride (10^{-4} M) increases the SCC across larval-type skin, whereas 2) amiloride blocks, but ACh has no effect on, the SCC of adult-type skin (2, 26). In skins taken from tadpoles at the climax stages (stages XX–XXV) of metamorphosis (Fig. 1), the SCC was increased by amiloride until stage XXI and by ACh until stage XXII. An amiloride-blockable response started to become apparent at stages XXI–XXII. This

Fig. 3. Effects of ACh (A, C, and E; 1 mM) and Am (B, D, and F; 10^{-4} M) on SCC (µA/cm²) of tadpoles raised for 5–11 days with T₃ (A and B; 10^{-8} M), Aldo (C and D; 10^{-8} M), or Aldo + T₃ (E and F). Tadpoles were at stages X–XIII at the start time. Effect of ACh or Am is expressed as ΔSCC (absolute difference between maximum response and baseline SCC). Open columns, increase in SCC; filled columns, no change in SCC (changes of <0.1 µA/cm²); hatched columns, decrease in SCC. Bars show means ± SE. Numbers in parentheses indicate number of experiments in which each response appeared.
result suggests that the functional replacement of the larval type of bullfrog epidermis by the adult type begins at stages XXI–XXII during natural metamorphosis.

ACh and amiloride responses in the skin of tadpoles raised with Aldo, T₃, or Aldo + T₃. Tadpoles were raised (starting at stages X–XIII) for 5–11 days in the presence of Aldo (Aldo; 10⁻⁶ M), T₃ (10⁻⁸ M), or Aldo + T₃. No forelimbs appeared in these tadpoles, except in one tadpole raised with T₃ for 11 days. The ACh and amiloride effects on the SCC were then investigated (Figs. 2 and 3).

The tadpoles raised in tap water for 11 days (to provide control larvae) reached stages XI–XIV. In these, the SCC was 0.50 ± 0.11 µA/cm² (n = 9), and the ACh- or amiloride-induced increase in the SCC (∆SCC) was 1.25 ± 0.49 (7/9 cases) or 0.77 ± 0.35 µA/cm² (6/9 cases), respectively. The ratio of tail length to total body length (T/B) in these larvae was 0.663 ± 0.007.

The SCC of tadpoles raised with T₃ for 5–11 days (by which time they had reached around stages X–XIII) was higher than that of the controls (P < 0.01). ACh increased this SCC, and amiloride did not decrease it in any tadpole.

The SCC of tadpoles raised with Aldo for 5–11 days (by which time they had reached around stages XV–XVII) was higher than that of the controls (P < 0.01). Amiloride increased this SCC, and ACh did not decrease it in any tadpole.

The SCC of tadpoles raised with Aldo + T₃ for 5–11 days (by which time they had reached around stages XVIII–XIX) was also higher than that of the controls (P < 0.001). Amiloride decreased this SCC in some 5–11-day-treated larvae (2/15 cases), and in most 9–11-day-treated larvae (8/8 cases). ACh did not increase the SCC in any larvae treated for 11 days with Aldo + T₃.

Thyroid hormone-treated animals are sometimes morphologically abnormal, with the tail length decreasing before the appearance of the forelimbs (7). The T/B ratio in larvae treated for 11 days with T₃, Aldo, or Aldo + T₃ was not significantly different from that of the controls (P > 0.08), indicating that abnormal tail regression did not occur under our conditions.

In tadpoles raised with Aldo + T₃, the amiloride-blockable SCC developed before the appearance of the forelimbs. This result is consistent with our hypothesis that there is a suppression mechanism(s) blocking the action of Aldo on the development of the amiloride-blockable SCC in tadpoles in vivo and that this mechanism(s) may be removed by the increase in thyroid hormone that occurs from stages XVIII–XIX onward (see Introduction).

Current fluctuation (noise) analysis of the SCC of tadpoles raised with Aldo + T₃. Amiloride-blockable Na⁺ channels contribute to the SCC across bullfrog skin (1, 19). We used current fluctuation analysis (noise analysis) of the SCC [which allows a quantitative analysis of the properties of a given channel (5, 7, 17)] to determine whether the amiloride-blockable Na⁺ channel that develops under Aldo + T₃ treatment in tadpoles in vivo is the same as the adult-type channel that develops in the skin of the naturally metamorphosed young adult (froglet) and in the EDTA-treated skin of tadpoles cultured with Aldo.

In the absence of amiloride, the spectrum was dominated by 1/ƒ noise. In contrast, in the presence of amiloride, a single Lorentzian component was clearly discernible (data not shown). The average corner frequency of the Lorentzian component is plotted against the corresponding amiloride concentration in Fig. 4. The characteristics of the channel, such as the single-channel current (i), the blocking (Kᵦᵦ) and unblocking rate coefficient for amiloride (Kᵦᵦ) determined from a least-squares linear regression of the 2πfᵦ values, and the apparent Kᵦᵦ are summarized in Table 1. The mean values obtained for i, Kᵦᵦ, Kᵦᵦ, M, and Kᵦᵦ were not significantly different among the channels in the skins of Aldo + T₃-treated tadpoles, naturally metamorphosed froglets, and skins cultured with Aldo (P > 0.08). This suggests that the amiloride-blockable Na⁺ channel developed in tadpoles raised with Aldo + T₃ and in skins cultured with Aldo really is of the adult type.


downloaded from http://ajpregu.physiology.org/ by 10.220.33.2 on October 6, 2016
Table 1. Summary of characteristics of skin of tadpoles raised with Aldo + T$_3$, froglet skin, and EDTA-treated tadpole skin cultured with Aldo

<table>
<thead>
<tr>
<th></th>
<th>TEP, mV</th>
<th>SCC, µA/cm$^2$</th>
<th>R, kΩ·cm$^2$</th>
<th>$I_{\text{Na(max)}}$, µA/cm$^2$</th>
<th>$I_{\text{i}}$, pA</th>
<th>$M_0$, channels/µm$^2$</th>
<th>$K_{\text{m}}$, s$^{-1}$·µM</th>
<th>$K_{\text{d}}$, s$^{-1}$</th>
<th>$K_m$, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldo + T$_3$ (n = 10)</td>
<td>5.2 ± 0.8*</td>
<td>5.9 ± 0.9*</td>
<td>0.90 ± 0.04</td>
<td>4.6 ± 0.8*</td>
<td>0.66 ± 0.16</td>
<td>0.10 ± 0.02*</td>
<td>11.4 ± 0.7</td>
<td>14.7 ± 1.9</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Froglet skin (n = 22)</td>
<td>11.7 ± 1.6</td>
<td>11.6 ± 1.6</td>
<td>1.04 ± 0.10</td>
<td>10.0 ± 1.6</td>
<td>0.53 ± 0.04</td>
<td>0.27 ± 0.05</td>
<td>11.0 ± 0.9</td>
<td>15.6 ± 2.4</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Cultured skin (n = 10)</td>
<td>13.2 ± 3.2</td>
<td>14.6 ± 2.6</td>
<td>0.87 ± 0.09</td>
<td>13.7 ± 2.6</td>
<td>0.57 ± 0.04</td>
<td>0.34 ± 0.10</td>
<td>13.4 ± 0.8</td>
<td>11.1 ± 2.3</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of samples used in each experiment. Tadpoles (stages IX–XIII) were raised with aldosterone (Aldo; 10$^{-6}$ M) + T$_3$ (10$^{-6}$ M) for 9–11 days. Froglet skin is from dorsal skin of 2- to 3-wk-old young adults from stage XXV (naturally metamorphosed). For cultured skin, EDTA-treated dorsal skin from tadpoles (stages XI–XV) was cultured with Aldo (10$^{-6}$ M) for 2 weeks. TEP, transepithelial potential difference; SCC, short-circuit current; R, skin resistance; $I_{\text{Na(max)}}$, maximum level of amiloride-sensitive SCC (total SCC – amiloride-insensitive SCC); $I_{\text{i}}$, single-channel current; $M_0$, density of amiloride-blockable Na$^+$ channels; $K_{\text{m}}$, blocking rate coefficient for amiloride; $K_{\text{d}}$, unblocking rate coefficient for amiloride; $K_m$, apparent dissociation constant ($K_m = K_{\text{d}}/K_0$). *Significantly different from corresponding values for froglet skin and cultured skin (P < 0.05).

increase in the endogenous thyroid hormone level that starts at stages XVIII–XIX.

When tadpoles at stages IX–XIII were raised with Aldo (10$^{-6}$ M) for 3–4 wk, the ACh-stimulated response was still present in most (7/8) cases, but an amiloride-blockable SCC developed in 5/8 stage-XIX larvae (Table 2). No forelimbs had developed in these larvae. In tadpoles at the stage at which the forelimbs appear (0- to 1-day-old larvae at stage XX), the SCC decreased by amiloride in all cases (7/7 cases). These results strongly support our hypothesis. The values for T/B ratio in tadpoles at those stages (stages XI and XX) were not significantly different from the T/B ratio of control tadpoles (raised in tap water; P > 0.9).

Non-EDTA-treated tadpole skin cultured with Aldo. The epidermis of larvae bullfrog skin is composed of apical cells, skin cells, and basal cells (20). Of these, the apical and skin cells are called larval cells, and these are assumed to disappear under the influence of thyroid hormone during the climax stages of metamorphosis. On the other hand, the basal cells are assumed to be the primordia of adult-type cells (31).

Because an amiloride-blockable SCC developed when EDTA-treated tadpole skin was cultured with Aldo for 2 wk, an amiloride-blockable SCC might have been expected to develop in vivo in the skin of tadpoles raised with Aldo for the same period (24, 27). However, this did not occur (25). There is actually a morphological difference between the tadpole skin used in the in vivo experiments and the EDTA-treated tadpole skin used in the in vitro experiments, because EDTA treatment removes both apical and skin cells (27). We cultured non-EDTA-treated skin from tadpoles at stages XII–XIV with Aldo (10$^{-6}$ M) for 2 wk; the SCC that developed was blocked by amiloride (Table 3). This suggests that the suppression mechanism(s) is not present in larval skins in culture in which apical and skin cells are intact at the start of the culture period and thus that these cells do not originate the suppression mechanism(s).

Immunocytochemistry of the skin. Human blood group antigen A is a specific molecular marker of the adult-type epidermis of bullfrog skin; that is, the adult-type skin reacts with antigen A-specific antiserum, whereas the larval-type skin does not (32). Actually, EDTA-treated tadpole skin cultured with Aldo and naturally metamorphosed froglet skin both react to the antisera (26, 27).

In the present study, the skin of tadpoles raised with Aldo + T$_3$ (in which an amiloride-blockable SCC developed before the appearance of the forelimbs) and non-EDTA-treated tadpole skin cultured with Aldo were both found to be immunocytochemically of the adult type (i.e., both reacted to the antigen A-specific antisera; Fig. 5). In both of these skin samples, the SCC was blocked by amiloride (data not shown).

Aldosterone has the potential to eliminate apical and skin cells in vitro, because all the apical cells and most of the skin cells disappeared when non-EDTA-treated skin was cultured with Aldo.

**DISCUSSION**

The present findings support our hypotheses 1) that the amiloride-blockable SCC that develops in tadpoles raised in Aldo + T$_3$ really is of the adult type, 2) that Aldo is crucial for the development of this SCC, and 3) that the effect of Aldo is blocked until a suppression
mechanism(s) is removed by the increases in T3 that normally occur at stages XVII–XIX.

The changes that occur in the effects of ACh and amiloride on the SCC across tadpole skin during the climax stages of metamorphosis suggest that the functional replacement of the larval cells of the epidermis by adult cells occurs at stages XXI–XXII. This conclusion is supported by morphological evidence that apical cells are present until stage XX while skein cells are present until stage XXII (11, 31). However, the present results suggest that our postulated suppression mechanism(s) does not originate from apical or skein cells. In tadpoles raised in T3, the ACh response remained intact, but the proportion of cases in which the SCC was increased by amiloride showed a decrease after day 7, and the proportion of amiloride-insensitive responses showed an increase after day 9. This suggests that these tadpoles were certainly on their way toward the climax stages. Indeed, in one animal treated with T3 for 11 days, not only did the left forelimb appear, but the SCC was blocked by amiloride. One explanation for this might be that prolonged treatment with T3 stimulates the secretion of Aldo (12) and that Aldo and T3 together then stimulate the development of an amiloride-blockable SCC, because amiloride never blocks the SCC of 0- to 1-day-old larvae (just after the left forelimb has appeared; stage XX larva) during natural metamorphosis (7, 22).

The larval-type (ACh-stimulated) response was still present in the skin of tadpoles raised with Aldo for 5–7 days; then it progressively disappeared, and the adult-type (amiloride-blockable) response progressively developed after 7–9 days of treatment. This suggests that a functional replacement of the larval type of epidermis by the adult type began at around days 7–9 of the period of Aldo treatment. However, the SCC across the skin of tadpoles raised with Aldo for 9–11 days was lower than that seen in skin cultured with Aldo alone (Table 3; P < 0.03). Possibly Na+ pump activity might have not been well developed in the former skin.

The properties of amiloride-blockable Na+ channels have been investigated by noise analysis, with amiloride as the channel blocker, in a number of previous studies (4, 5, 7, 17, 19). Generally, values of i of 0.3–0.6 pA, K10 values of 8–18 s^-1 ·µM^-1, and K10 values of 1–25 s^-1 have been reported for frog skin. The present values lie within or very close to those ranges.

Hillyard and Van Driessche (6) reported that the SCC across the skin of larvae at stage XIX or earlier is insensitive to a 24-h treatment with Aldo; that is, the hormone produced no significant change in SCC in such larvae, even though their metamorphosis had been stimulated by thyroid hormone. However, the skin of larvae at these stages is not actually insensitive to Aldo, because 1) an amiloride-blockable SCC developed when tadpole skin at stages XIII–XV was cultured with Aldo for at least 7 days (27), and 2) both the baseline SCC and the ACh-stimulated SCC in stage-XI to -XVI larvae were potentiated by Aldo when tadpoles were raised with the hormone for 2 wk (25). On the other hand, injection of Aldo (2 × 10^-7 M/kg body wt) every other day for 2 wk in stage-XIII to -XV larvae had no effect on SCC (23). Possibly in that study the Aldo might have been excreted, and its concentration might therefore not have been high enough for long enough to affect the SCC. Aldo may need to be present for >24 h.

### Table 3. Non-EDTA-treated tadpole skin cultured with Aldo

<table>
<thead>
<tr>
<th>TEP, mV</th>
<th>SCC, µA/cm²</th>
<th>R, kΩ cm²</th>
<th>ΔSCC (µA/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh, 1 mM</td>
<td>Am, 10^-4 M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inc</td>
<td>No</td>
<td>Dec</td>
<td>Inc</td>
</tr>
<tr>
<td>2.46 ± 0.95 (5)</td>
<td>7.33 ± 2.30 (5)</td>
<td>0.32 ± 0.06 (5)</td>
<td>0.31 ± 0.07 (4)</td>
</tr>
</tbody>
</table>

Values are means ± SE; numbers in parentheses indicate no. of experiments. Non-EDTA-treated skin from tadpoles at stages XII–XIV was cultured for 2 wk.

Fig. 5. Hematoxylin and eosin staining (A and B) and immunocytochemical staining (C and D) of skin. A, C: tadpoles raised in presence of Aldo (10^-6 M) + T3 (10^-8 M) for 11 days. B, D: non-EDTA-treated tadpole skin cultured with Aldo (10^-6 M) for 2 wk. Bar: 25 µm.
with its concentration continuously over some critical concentration before it can affect the SCC.

Use of an enormous dose of Aldo is necessary for the development of an amiloride-blockable SCC in vivo (27). The reason for this does not seem to be that Aldo binds to a glucocorticoid receptor rather than to a mineralocorticoid receptor, because the SCC developed in tadpole skin cultured with hydrocortisone and corticosterone was lower than that seen in skin cultured with Aldo at the same concentration (27 and M. Takada, unpublished data).

It is assumed that the amiloride-blockable Na\(^+\) channel involved in producing the amiloride-blockable SCC develops on granular cells, one of the adult-type cells (1). Granular cells develop at stage XVIII (11), a stage at which the amiloride-blockable Na\(^+\) channel has not yet developed during natural metamorphosis. The lag between these two events could be explained in at least two ways: 1) the endogenous concentration of Aldo is not high enough for the development of the amiloride-blockable Na\(^+\) channel before stage XVII in vivo, but it then increases at stages XVIII–XIX to a level suitable for channel development; or 2) at stage XVIII the action of Aldo on channel development is still suppressed by an as yet unknown mechanism(s), but this is removed by thyroid hormone when its endogenous level starts to increase at stages XVIII–XIX. In either scheme, the amiloride-blockable Na\(^+\) channel would develop some time after the appearance of granular cells in the course of natural metamorphosis. Either scheme would explain why the amiloride-blockable SCC has not developed at stage XVIII, and why Aldo, or even Aldo + T\(_3\), treatment would be ineffective at developing an amiloride-blockable SCC before stage XVIII (i.e., before the development of granular cells). In fact, in our view the weight of evidence now favors the idea that the two schemes operate together. If this is so, the increases in Aldo and thyroid hormone that occur at stages XVIII–XIX would act in concert to promote the development of an amiloride-blockable Na\(^+\) channel.

Prolactin is thought to maintain larval-type characteristics. However, the suppression mechanism(s) blocking the action of Aldo seems not to depend on prolactin, because thyroid hormone (which is believed to antagonize the action of prolactin) does not antagonize the action of prolactin on the Aldo-induced development of an amiloride-blockable SCC (28).

A three-step mechanism to explain the action of thyroid hormone on amphibian metamorphosis has been proposed by Kawai et al. (13) and Yoshizato (31). In their scheme, step I (tail bud stage up to TK stage IV) proceeds without thyroid hormone. In step II (up to stage XIX), basal cells (primordia of the adult-type cells) develop with the aid of a trace of thyroid hormone (\(>10^{-10}\) M). In step III (up to stage XXV), body skin is completely transformed into the adult-type skin under the influence of a higher level of thyroid hormone (\(>10^{-9}\) M as T\(_3\)). Thyroid hormone alone seems, however, to be insufficient for the development of the adult-type characteristics of the amphibian epithelium, at least in some cases (e.g., small intestine of Xenopus laevis or cultured larval Rana catesbeiana skin [8, 10, 27]). Our hypothesis, as outlined and supported in this paper, is consistent with the three-step model in that the role of a higher level of thyroid hormone in the last step could involve the removal of our postulated suppression mechanism(s). The identification of this unknown suppression mechanism(s) will be a matter for future experimentation.

**Perspectives**

Morphological studies of the amphibian small intestine and of body and tail skin have shown that the metamorphic fate of the epithelium is determined by the connective tissues (mesenchymal tissues) (15, 31). The enzymatically separated epithelium and connective tissues are recombined heterotypically. The body epithelium of the skin is transformed morphologically to tail-type epithelium, and the adult epithelium of the small intestine develops only in association with recombinant-containing connective tissues, suggesting that connective tissues are physically and/or chemically involved in the morphological development of epithelia (8–10, 15). On this basis, connective tissues are probably involved, too, in the functional development of adult-type characteristics, such as the amiloride-blockable SCC. An insulin-like factor or other endogenous factor(s) from connective tissue are possible candidates for involvement in the development of an adult-type epithelium, and for the development of the SCC in vivo, 1) thyroid hormone is unnecessary and 2) such an enormous dose of Aldo is necessary (when given alone) are questions that may be solved by such research.

The authors thank Dr. A. Arita (Professor of Physiology, Junior College of Saitama Medical School) for valuable comments on noise analysis. The authors also greatly appreciated the skill shown by M. Makimoto (Nihon Kohden) in designing and improving the SCC amplifier. The experiments described in this paper were conducted in accordance with the current Japanese law governing animal experimentation.

Address for reprint requests and correspondence: M. Takada, Dept. of Physiology, Saitama Medical School, Moroyama, Iruma-gun, Saitama, 350–0495 Japan (E-mail: makokam@saitama-med.ac.jp).

Received 26 Jan 1999; accepted in final form 7 Jun 1999.

**REFERENCES**


