Effect of calcium on development of amiloride-blockable Na⁺ transport in axolotl in vitro

Takada, Makoto, Hideko Yai, and Shinji Komazaki. Effect of calcium on development of amiloride-blockable Na⁺ transport in axolotl in vitro. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R69–R75, 1998.—The axolotl, Ambystoma mexicanum, which has no specific calcium-containing sieve layer in the dermis, provides useful material for the study of the effect of Ca²⁺ on the development of amiloride-blockable active Na⁺ transport across the skin of amphibians. We raised axolotls in thyroid hormone or aldosterone or cultured the skin with corticoid plus one of several Ca²⁺ concentrations and found that 1) although the short-circuit current (SCC) was increased by both aldosterone and 3,3',5-triiodo-L-thyronine in vivo, only corticoid was necessary for such an increase in vitro; 2) the development of the SCC in vitro was both corticoid and Ca²⁺ dependent, because the SCC was well developed with over 100 µM Ca²⁺ but not with under 10 µM Ca²⁺ in the presence of corticoid, nor even with 300 µM Ca²⁺ without corticoid; and 3) Ca²⁺, but not corticoid, was necessary for the formation of cell-to-cell junctions, because the resistance of the skin was well developed with 300 µM Ca²⁺ without corticoid.

An additional advantage of the axolotl as an experimental animal is that active Na⁺ transport does develop without thyroid hormone in the bullfrog, the possibility cannot be excluded that endogenous thyroid hormone in the larval skin may exert a synergistic action with corticoid on the development of this type of transport. Consequently, the axolotl is an ideal species for the investigation of the effect of Ca²⁺ on the development of this type of transport in vitro using the skin of bullfrog tadpoles because the sieve layer located in their dermis contains so much calcium (8, 11). In contrast, the axolotl, Ambystoma mexicanum, has no sieve layer in its dermis, so this type of analysis can be conducted without the confusing effect of the calcium already present in the dermis.

The axolotl has an additional advantage as an experimental animal. Although we now believe that active Na⁺ transport develops without thyroid hormone in the bullfrog, the possibility cannot be excluded that endogenous thyroid hormone in the larval skin may exert a synergistic action with corticoid on the development of this type of transport. Consequently, the axolotl is an ideal species for the investigation of the effect of Ca²⁺ and of the interaction between corticoid and thyroid hormone on the development of amiloride-blockable SCC. Use of the axolotl in the present study also allowed us to determine whether the development of amiloride-blockable SCC, which can be induced by corticoid alone in R. catesbeiana (Anura), can be induced in the same way in the axolotl (Urodele).

MATERIALS AND METHODS

Animals. Larval A. mexicanum were purchased from a local animal supplier in Hamamatsu, Shizuoka, Japan, and maintained for 3 wk in tap water (control larvae) or in tap water supplemented either with 3,3',5-triiodo-L-thyronine (T₃) (10⁻⁵ M; T₃-treated larvae) or with aldosterone (Aldo; 5 × 10⁻⁷ M; Aldo-treated larvae). The water in the aquarium was changed every 3 days. Animals were fed with Limnodrilus (annelid) or Profit (food substance for eels made by Nippon Haigoshiryō, Yokohama, Japan).

Culture of skin. Intact larvae were anesthetized with ice water containing 0.05% MS-222 (Sankyo, Tokyo, Japan), and a portion of the body or head skin was dissected out. The culture method used for larval skin was similar to that described by Takada et al. (20). In brief, the skin was washed with 70% ethanol and then with Ca²⁺- and Mg²⁺-free saline...
R70  EFFECT OF CALCIUM ON Na+ TRANSPORT IN AXOLOTL

(CMFS) and transferred to CMFS containing 2.5 mM EDTA; this treatment leaves only the innermost layer intact. Next, the skin was washed with normal saline and transferred to tissue culture medium (see Culture medium). The skin was cultured in a humidified atmosphere of 5% CO2 and 95% room air at 24°C for a number of weeks (see RESULTS).

Culture medium. One of two kinds of RPMI-1640 solution (Gibco, Grand Island, NY), containing either 424 µM Ca2+ or 0.45 µM Ca2+, was used as the starting solution for the culture medium. Both solutions were diluted to 70% with distilled water and supplemented with 16.7 mM NaHCO3, 10 mM HEPES (pH 7.4), 100 IU/ml penicillin, and 100 µg/ml streptomycin. Glutamine (1.4 mM) was also added to the RPMI-1640 solution containing 0.45 µM Ca2+ so that its overall concentration of solutes was the same as that of the RPMI-1640 solution containing 424 µM Ca2+ (which contained 1.4 mM glutamine). Although it is conceivable that development of an amiloride-blockable SCC is affected by the concentration of glutamine in the medium, we know of no evidence that this is the case in the axolotl. As a result of the dilution to 70%, the Ca2+ concentration in the first medium was ~300 µM (normal RPMI) and that in the second medium was 0.32 µM (low-Ca RPMI). To produce 10 µM Ca2+, 100 µM Ca2+-containing media, Ca(NO3)2 was added to the low-Ca RPMI or to normal RPMI. The skin was cultured in the above media with or without 1) 5 × 10−7 M Aldo, 2) Aldo + 5 × 10−7 M hydrocortisone + 5 × 10−7 M corticosterone (Mix), and 3) 10−8 M T3.

Measurement of PD, SCC, and R of skin. Freshly dissected skin from whole animals (taken under anesthesia induced with ice water supplemented with MS-222) or cultured skin samples were mounted in a Ussing-type chamber using silicone gaskets (inner diameter 5 mm) to minimize edge damage. Both sides of the skin were bathed in aerated Ringer solution containing (in mM) 310 NaCl, 2 KC1, 1 CaCl2, 10 glucose, and 10 Tris at pH 7.2 and allowed to equilibrate for 1 h. To produce an Na+-free solution, all NaCl was replaced by choline-chloride. The potential difference (PD) across the skin, the SCC, and the skin resistance (R) were measured using the method of Takada et al. (20, 21). The fluid R was compensated. When required, amiloride was applied to the apical side and ouabain to the basolateral side of the skin.

Light microscopy. After the culture period, the skin was fixed with 10% formalin, embedded in paraffin, and sectioned at 8-µm thickness. The sections were stained with hematoxylin and eosin and viewed under a light microscope.

Statistical analysis. Values are expressed as means ± SE. Differences were analyzed using Dunnett's one-tailed t-test, Student's t-test, or Welch's test; they were taken as significant when P < 0.05.

RESULTS

SCC across the skin. In the present study, the gills of animals raised in T3 (10−8 M) for 3 wk (T3-treated larvae) were reduced to 2.2 ± 0.86 from 10.8 ± 0.58 mm in length (mean ± SE; n = 5, significantly different at P < 0.05), showing that this treatment had triggered metamorphosis. On the other hand, the gills of animals raised in Aldo (5 × 10−7 M; Aldo-treated larvae) did not show any detectable regression. In both the T3-treated larvae and the Aldo-treated larvae, the SCC across the skin was significantly higher than in the control larvae, indicating that an increase in SCC can be induced by either T3 or Aldo in vivo (Table 1).

| Table 1. PD, SCC, and R across skin taken from whole animals and across cultured skin |
|------------------|--------|-----------------|-------------|
|                  | n     | PD, mV          | SCC, µA/cm2 | R, kΩ·cm2 |
| Skin from whole animals |       |                 |             |           |
| Control larvae    | 7     | 0.90 ± 0.34     | 1.36 ± 0.49 | 0.63 ± 0.09 |
| T3 treated        | 4     | 11.1 ± 5.58     | 5.10 ± 1.34 | 1.21 ± 0.31 |
| Aldo treated      | 20    | 19.5 ± 0.26     | 2.84 ± 0.25 | 0.69 ± 0.05 |
| Cultured skin     |       |                 |             |           |
| EDTA treated      | 4     | 0               | 0          | 0.26 ± 0.02 |
| RPMI-1640 1        | 12    | 0.09 ± 0.06     | 0.09 ± 0.06 | 0.85 ± 0.11 |
| T3                 | 11    | 1.48 ± 0.09     | 0.90 ± 0.28 | 1.15 ± 0.25 |
| Aldo               | 6     | 10.8 ± 1.5      | 6.85 ± 0.72 | 1.58 ± 0.22 |
| Mix                | 20    | 10.2 ± 2.4      | 6.54 ± 1.09 | 1.55 ± 0.23 |
| Mix + T3           | 23    | 8.54 ± 2.45     | 6.65 ± 1.34 | 0.97 ± 0.13 |

Values are means ± SE; n = no. of experiments. In skin from whole animals, control larvae were raised in tap water, 3,3′,5-triiodo-l-thyronine (T3)-treated larvae were raised in T3 (10−8 M) for 3 weeks, and aldosterone (Aldo)-treated larvae were raised in Aldo (5 × 10−7 M) for 3 weeks. In cultured skin, EDTA-treated skin was cultured in normal RPMI-1640 under the following hormonal conditions for 3 weeks: EDTA treated, isolated larval skin just after EDTA treatment and before culture (see MATERIALS AND METHODS); RPMI-1640, normal RPMI-1640 (hormone free); T3, T3 (10−8 M); Aldo, Aldo (5 × 10−7 M); Mix, corticoid mixture (5 × 10−7 M Aldo + 5 × 10−7 M hydrocortisone + 5 × 10−7 M corticosterone); Mix + T3, Mix (see above) + T3 (10−8 M). PD, potential difference; SCC, short-circuit current; R, resistance. Superscript b indicates significant difference from superscript a (P < 0.05) (Dunnett's 1-tailed t-test).

To test whether the development of the SCC can be induced by corticoid alone in vitro, EDTA-treated axolotl skin was cultured under a variety of hormonal conditions for 3 wk. As shown in Table 1, in skin cultured in normal RPMI-1640 supplemented with Aldo, Mix, or Mix + T3, the SCC was significantly higher than in the control larvae. By contrast, in skin cultured in normal RPMI-1640 supplemented with T3, the SCC was not significantly different from that of the controls, nor was it significantly different from the SCC in EDTA-treated skin or in skin cultured in normal RPMI without any hormone.

The conclusion that can be drawn from the above results is similar to that reported for cultured larval bullfrog skin, that is, that an amiloride-blockable SCC can develop under the influence of corticoid alone (20). It is clear that a synergistic action of corticoid and endogenous thyroid hormone is not necessary for the development of the SCC because the axolotl has no endogeneous thyroid hormone.

Time course of development of SCC. The SCC of skin cultured for 2 wk with Mix + T3 was greater than that of skin cultured with Mix alone (Fig. 1). However, after a 3-wk culture, the skin had developed about the same SCC regardless of the culture medium used (see also Table 1). Thus Mix alone eventually produced the same enhancement of the SCC as Mix + T3, but its effect occurred more slowly.

Effects of amiloride, ouabain, and Na+-free medium on SCC. Table 2 shows the effects of amiloride (10−4 M), ouabain (10−5 M), and Na+-free Ringer (1) on the SCC across skin taken from control larvae, T3-treated larvae, and Aldo-treated larvae (no data for Na+-free Ringer) and 2) on the SCC across skin cultured with
Effects of amiloride, ouabain, and Na-free conditions on SCC

Table 2. Effects of amiloride, ouabain, and Na-free conditions on SCC

<table>
<thead>
<tr>
<th>Condition</th>
<th>SCC (µA/cm²)</th>
<th>Amiloride (10⁻⁴ M)</th>
<th>Ouabain (10⁻⁵ M)</th>
<th>Na-free Ringer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td>Skin from whole animals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control larvae</td>
<td>1.04 ± 0.10</td>
<td>0.56 ± 0.90*</td>
<td>1.71 ± 0.78</td>
<td>0.79 ± 0.07</td>
</tr>
<tr>
<td>(16)</td>
<td>(5)</td>
<td></td>
<td>(3)</td>
<td>(5)</td>
</tr>
<tr>
<td>T₃ treated</td>
<td>6.43 ± 2.15</td>
<td>1.15 ± 1.26*</td>
<td>7.14 ± 0.67</td>
<td>1.18 ± 0.51*</td>
</tr>
<tr>
<td>(5)</td>
<td></td>
<td></td>
<td>(3)</td>
<td>(3)</td>
</tr>
<tr>
<td>Aldo treated</td>
<td>2.51 ± 0.75</td>
<td>0.26 ± 0.34*</td>
<td>2.64 ± 0.57</td>
<td>0.76 ± 0.26*</td>
</tr>
<tr>
<td>(5)</td>
<td></td>
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<td>(5)</td>
<td>(5)</td>
</tr>
<tr>
<td>Cultured skin Mix</td>
<td>6.64 ± 2.25</td>
<td>1.73 ± 1.09*</td>
<td>8.61 ± 2.11</td>
<td>4.59 ± 0.96*</td>
</tr>
<tr>
<td>(14)</td>
<td>(7)</td>
<td></td>
<td>(7)</td>
<td>(7)</td>
</tr>
<tr>
<td>Mix + T₃</td>
<td>7.38 ± 2.51</td>
<td>1.68 ± 1.31*</td>
<td>12.2 ± 3.59</td>
<td>5.31 ± 1.24*</td>
</tr>
<tr>
<td>(11)</td>
<td>(5)</td>
<td></td>
<td>(5)</td>
<td>(5)</td>
</tr>
</tbody>
</table>

Values are means ± SE with no. of experiments in parentheses. Before, before application of amiloride or ouabain or Na-free Ringer; after, after application of amiloride on apical side for 5–10 min, ouabain on basolateral side for 30–40 min, or Na-free Ringer on both sides for 5–10 min. *P < 0.05 vs. before (Student’s t-test).
To investigate in more detail the relationship between the effects of Ca\textsuperscript{2+} and Mix on the development of an SCC and R, EDTA-treated skin was cultured for 6 wk or more under a variety of conditions (i.e., in various Ca\textsuperscript{2+} concentrations with or without Mix; Fig. 3). As stated above, an SCC did not develop when EDTA-treated skin was cultured with 0.3 µM Ca\textsuperscript{2+} + Mix for 3 wk (Fig. 2). After an additional 3-wk culture with 300 µM Ca\textsuperscript{2+} + Mix, the skin still did not develop an SCC or R (total 6 wk; Fig. 3, A, B, and C). On the other hand, although an SCC did not develop in skin cultured with 10 µM Ca\textsuperscript{2+} + Mix for 3 wk (Fig. 2), an additional 3-wk culture with 300 µM Ca\textsuperscript{2+} + Mix resulted in a considerable SCC and R (total 6 wk; Fig. 3, A, B, and C). Although only R developed (and SCC did not) without Mix, even with 300 µM Ca\textsuperscript{2+} present for 3 or 6 wk (Table 1 and Fig. 3, A, B, and C), an additional 3-wk culture with 300 µM Ca\textsuperscript{2+} + Mix led to the development of a large SCC and a large R (total 6 or 9 wk; Fig. 3, A, B and C). Furthermore, neither SCC nor R developed in skin that was cultured first with 300 µM Ca\textsuperscript{2+} without Mix and then with 10 µM Ca\textsuperscript{2+} with Mix (total 6 wk; Fig. 3, A, B, and C).

Light microscopic observation of the skin. To examine whether morphological adult-type characteristics develop at the same time as the functional ones, skin samples were examined histologically. Leydig cells, which are large and granule rich and characterize the larval epidermis of urodeles, were present in the control skin of the axolotl (Fig. 4a) (5, 7, 24) and also in the skin of Aldo-treated larvae (data not shown). However, few were present in the skin of T3-treated larvae (Fig. 4f). EDTA treatment left only the innermost layer of the skin (Fig. 4b). It may be that the cells of this layer are precursors of the other epidermal cells, as they are in the case of bullfrog skin. If this is so, we might expect that a stratified epidermis, such as that seen in the skin of T3-treated larvae (but not Leydig cells, such as are seen in the control skin), will develop when EDTA-treated skin is cultured in normal RPMI supplemented with Mix, because an amiloride-blockable SCC is well developed in such skin (see Table 2).

In fact, Fig. 4d shows that when cultured with Mix, EDTA-treated skin did indeed develop a stratified epidermis and had no Leydig cells, as expected. In terms of its morphology, skin cultured with Mix + T3 (Fig. 4e) was much the same as skin cultured with Mix alone (Fig. 4d). We also expected that skin cultured with normal RPMI alone might possess a larval-type epidermis rather than the metamorphosed-type (stratified) epidermis, because the SCC across such skin is much lower than that across control skin. Actually, the skin was well maintained and showed stratification, but no Leydig cells were observed (Fig. 4e). Thus axolotl skin cultured with normal RPMI alone (without corticoid) developed cells that were morphologically like the metamorphosed type, even though functionally it was still of the larval type with only a low SCC. In contrast, skin of Aldo-treated larvae, which was morphologically like the larval type, had developed metamor-

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Fig. 2. Effect of Ca\textsuperscript{2+} on development of SCC and skin resistance (R). EDTA-treated skin was cultured with Mix + 0.3, 10, 100, 300, or 1,000 µM Ca\textsuperscript{2+} for 3 wk. A: effect of Ca\textsuperscript{2+} on SCC and R. B: Lineweaver-Burk plot of Ca\textsuperscript{2+} vs. SCC. C: Lineweaver-Burk plot of Ca\textsuperscript{2+} vs. R. In Lineweaver-Burk plots, maximum value of SCC or R is given by reciprocal of intercept on y-axis (i.e., intercept represents 1/maximum SCC or 1/maximum R). Ca\textsuperscript{2+} concentration required for a one-half-maximum effect (K\textsubscript{m}) is obtained by taking the reciprocal of the intercept on the x-axis and ignoring the sign (i.e., intercept represents −1/K\textsubscript{m}). Values are means ± SE. Numbers in parentheses show number of experiments. \*P < 0.01 vs. 10 µM Ca\textsuperscript{2+} (Dunnett’s 1-tailed t-test).
DISCUSSION

In the present study, amiloride attenuated the SCC of the control axolotl skin. In addition, Aldo treatment in vivo induced an increase in the SCC. Because the axolotl has no endogenous thyroid hormone, this is in line with our hypothesis (based on a study of bullfrog tadpole skin in vitro) that the development of an amiloride-blockable SCC across bullfrog skin is not the direct result of an action of thyroid hormone (20). Although, in the axolotl, in vivo T3 treatment led to the development of an amiloride-blockable SCC, this may be explained, as it is in the bullfrog (12), by T3 stimulating the secretion of Aldo, which in turn induces the development of the SCC.

Thyroid hormone enhanced the corticoid-induced SCC in the axolotl after 2 wk in culture but not after 3 wk in culture. Thyroid hormone also enhances the hydrocortisone-induced biosynthesis of 63-kDa keratin, a molecular marker of the metamorphosed-type epidermis in Xenopus (14–16). However, the development of a corticoid-induced amiloride-blockable SCC was not enhanced by thyroid hormone in bullfrog skin in vitro (20). Possibly, the synergy between the actions of T3 and corticoid may vary with the species and with the culture conditions.

A number of pieces of evidence indicate that Ca2+ has some involvement in the morphological and functional development of the epithelium in a variety of species. The proliferation of epidermal cells in the mouse and in amphibians was stimulated when they were cultured with less than 100 µM Ca2+, but differentiation (such as cornification) was inhibited (9, 14, 15). In contrast, proliferation was inhibited but differentiation was stimulated when these cells were cultured with over 100 µM Ca2+. Furthermore, Ca2+ is concerned with the formation of cell polarity and cell-to-cell tightening in MDCK cells (1, 2, 6, 23). These results led us to think that Ca2+ may be involved in the development of an amiloride-blockable SCC in amphibian skin. Thus morphological development of the epidermis might be expected in EDTA-treated axolotl skin cultured with under 100 µM Ca2+, whereas the development of an amiloride-blockable SCC might be expected in skin cultured with over 100 µM Ca2+. In fact, functional development (an increase in the amiloride-blockable SCC) was stimulated when EDTA-treated axolotl skin was cultured with Ca2+ at concentrations of over 100 µM, as we expected. However, morphological development of the epidermis was not stimulated in skin cultured with 0.3 µM or 10 µM Ca2+, but it was stimulated with concentrations of over 100 µM Ca2+, just as functional development was.

The development of higher SCC values in media containing higher levels of Ca2+ may indicate that tightening of cell-to-cell junctions is necessary for the proper development of an amiloride-blockable SCC. Indeed, even if Na+ channels and the Na+ pump...
develop, the presence of loose junctions would decrease the net flux of Na\(^+\) through the low shunt resistance \((R_S)\) of the skin, which would then fail to develop a full SCC (3, 13). Actually, a high transepithelial \(R\) is not formed in low-Ca\(^{2+}\) concentrations but is formed in high-Ca\(^{2+}\) concentrations, the one-half-maximum effect (in terms of the formation of cell-to-cell junctions) being produced by 50 µM Ca\(^{2+}\) in MDCK cells (23). In the present study, the one-half-maximum effect of Ca\(^{2+}\) on the development of \(R\) (which is due to epidermal cells) was 359 µM (Fig. 2C). This discrepancy may reflect differences in the development of cell-to-cell junctions between epithelial MDCK cells and the epidermal cells of the axolotl. Development of amiloride-blockable Na\(^+\) channels would be expected to lead to a decrease in total skin \(R\); in fact, \(R\) can actually increase. Possibly, this may be due to an increase in \(R_S\) (17). Whether the \(R_S\) is localized in cell-to-cell tight junctions or in other sites needs to be clarified using vibrating microprobe analysis.

An amiloride-blockable SCC might be expected to develop simultaneously with the adult features of the skin in the axolotl, because the SCC develops together with the adult features of the epidermis in the skin of the bullfrog (19, 20). Actually, an amiloride-blockable SCC was already present to a small extent in the intact skin of the axolotl, but an increase in the SCC would be expected to occur with the morphological development of adult features, that is, the disappearance of Leydig cells, which are a morphological marker of the larval-type epidermis of the axolotl (5, 24). In fact, as shown in Fig. 4, few Leydig cells were observed in the skin of T\(_3\)-treated larva or in skin cultured with corticoid (i.e., such skins were morphologically much the same as adult skin), and these skins did indeed show an increased SCC (Table 1). However, in skin cultured with normal RPMI, Leydig cells were not observed, yet there was no increase in the SCC. Thus in the axolotl, the disappearance of larval-type cells is not necessarily accompanied by an increase in the SCC. This dissociation between the development of an adult-type morphology and the development of an amiloride-blockable SCC (two events that occur concomitantly in the bullfrog) will clearly require further investigation.

The present study has confirmed that the development of an amiloride-blockable SCC can be induced by corticoid alone in vitro in the skin of the axolotl (Urodela), just as it can in the skin of R. catesbeiana (Anura). Moreover, the present results suggest that, at least in the axolotl, both calcium and corticoid are necessary for the full development of an amiloride-blockable SCC.

**Perspectives**

In bullfrog skin, the effect of Aldo on the development of an amiloride-blockable SCC differs between the in vitro and in vivo conditions (20). The SCC develops in the presence of Aldo alone in vitro but not in vivo, suggesting the existence of an inhibitory mechanism preventing Aldo from developing the transport in vivo. In contrast, in the axolotl no such inhibitory mecha-
nism seems to exist, because Aldo is also effective in vivo. Our future work will include a comparative study of this inhibitory mechanism in anurans and urodèles. Calcium participates in the docking and fusion mechanisms by which vesicles release transmitters at synaptic terminals. It is conceivable that similar mechanisms are involved in the development of cell polarity in epithelia. If so, Ca\textsuperscript{2+} may modulate the effect of Aldo by virtue of its induction of cell polarity. This has already been explored in the DISCUSSION.

The authors thank M. Suzuki for the constant supply of axolotls. They also thank M. Shibashi and M. Kasai for statistical analysis and for drawing figures.

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Received 3 December 1997; accepted in final form 3 March 1998.

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