

## PROLACTIN ANTAGONIZES THE CORTICOID-PROMOTED DEVELOPMENT OF ADULT-TYPE EPIDERMIS IN CULTURED LARVAL BULLFROG SKIN

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### Summary

EDTA-treated larval bullfrog skin, in which apical and skein cells had been removed and only basal cells remained, was cultured in one of four media. These contained either aldosterone (Aldo) or a mixture of Aldo, hydrocortisone (HC) and corticosterone (C), each either supplemented with prolactin (PRL) or lacking PRL. Skin cultured with Aldo alone or with the corticoid mixture (Aldo+HC+C) developed an adult-type epidermis: (i) both types of skin reacted to human blood group antigen A, a marker for the adult-type epidermis of bullfrog skin; (ii) amiloride decreased the short-circuit current  $I_{sc}$  in these skin preparations, but acetylcholine (ACh) had no effect on the  $I_{sc}$ . It seemed to make little difference to the results whether

the skin was cultured with Aldo or with the corticoid mixture. PRL antagonized the action of Aldo and induced the development of a larval-type epidermis in both skin preparations: (i) the skin preparations did not react to human blood group antigen A; (ii) acetylcholine and amiloride each stimulated  $I_{sc}$  in these preparations. Since ACh and amiloride each stimulated the  $I_{sc}$  in skin with apical cells, ACh/amiloride-stimulated channels may be located on these cells.

Key words: prolactin, aldosterone, larval bullfrog, *Rana catesbeiana*, cultured skin, epidermis, development.

### Introduction

The epidermis of the skin of the larval bullfrog is composed of 4–5 layers. The outermost layer is a single layer of apical cells and under this there are 2–3 layers of skein cells. The innermost layer is a layer of basal cells. These cells are all larval-type cells, and both apical and skein cells undergo cell death by the action of thyroid hormone during the climax stages of metamorphosis (Nishikawa and Yoshizato, 1986; Robinson and Heintzelman, 1987). In contrast to that of the larva, the epidermis of the adult bullfrog skin is composed of 6–7 layers making up the stratum corneum, stratum granulosum and stratum germinativum, from the apical to the basal side (Farquhar and Palade, 1965). Differentiation from larval-type to adult-type epidermis originates from the basal cells in the larval epidermis. These are the precursor cells of the adult-type epidermis and they promote the development of adult-type cells in response to thyroid hormone (Nishikawa and Yoshizato, 1986; Robinson and Heintzelman, 1987).

Developmental changes in the morphology of the skin are accompanied by changes in its functions. A typical example is the development of an amiloride-blockable active  $\text{Na}^+$ -transport system across the skin. In tadpole skin, acetylcholine (ACh) and amiloride both enhance the short-circuit current ( $I_{sc}$ ) by activating nonselective cation channels (Cox, 1993). In adult frog skin, in contrast, ACh no longer has any effect on

$I_{sc}$ , and amiloride actually inhibits the  $I_{sc}$  caused by active  $\text{Na}^+$  transport, for which channels develop during the climax stages of metamorphosis (Kirschner, 1955; Cox and Alvarado, 1979; Takada, 1985). This amiloride-blockable active  $\text{Na}^+$ -transport system was thought to be induced by thyroid hormone, since it is known that developmental changes in the epidermis are induced by thyroid hormone. However, Takada *et al.* (1995) have recently found that the amiloride-blockable active  $\text{Na}^+$ -transport system develops under the action of corticoid, in the absence of thyroid hormone. They cultured EDTA-treated larval skin, in which apical and skein cells had been removed and only basal cells remained, in a medium containing corticoid but not thyroid hormone. In such skin, they showed that the development of active  $\text{Na}^+$  transport, measured as  $I_{sc}$ , still occurred. Such EDTA-treated larval skin was thought by us to be appropriate for investigating the effects of hormones on the morphogenesis of bullfrog epidermis, since only basal cells remain in the preparation. Using a method based on that of Takada *et al.* (1995), we have identified a correlation between the morphology and functioning of EDTA-treated larval skin. The skin was cultured with either aldosterone (Aldo) or with a mixture of Aldo, hydrocortisone (HC) and corticosterone (C), each supplemented with prolactin (PRL) or lacking PRL. We used the response of the  $I_{sc}$  to ACh and

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amiloride as a functional marker and human blood group antigen A as an immunocytochemical marker.

## Materials and methods

### Animals

Tadpoles of *Rana catesbeiana* were purchased from a local animal supplier in Misato City, Saitama, Japan. They were maintained in tap water and fed with boiled spinach, the water in the aquarium being changed every 2 days. After developmental stage XXI, the tadpoles were moved to another aquarium and maintained in shallow water without feeding. The stages were determined by reference to the work of Taylor and Kollros (1946).

### Culture of dorsal skin

Details of the method of dissection and culture of the dorsal skin of tadpoles have been published elsewhere (Takada *et al.* 1995). In brief, tadpoles were anaesthetized with iced water and the dorsal body skin dissected away. The skin was washed with 70% ethanol, then with  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free saline (CMFS), and transferred to CMFS containing  $2.5 \text{ mmol l}^{-1}$  EDTA to remove apical cells. Next, the skin was washed with normal saline and transferred to tissue culture medium (see below). The skin was cultured in a humidified atmosphere of 5%  $\text{CO}_2$  and 95% room air at  $24^\circ\text{C}$  for 1–2 weeks.

### Culture medium

RPMI solution was prepared as follows and used as the culture medium: RPMI-1640 (Gibco, Grand Island, NY, USA) was diluted to 70% with distilled water, and supplemented with  $16.7 \text{ mmol l}^{-1}$   $\text{NaHCO}_3$ ,  $10 \text{ mmol l}^{-1}$  Hepes (pH 7.4),  $100 \text{ i.u. ml}^{-1}$  penicillin and  $100 \mu\text{g ml}^{-1}$  streptomycin. The skin was cultured in the above medium with (i) corticoid mixture [Mix:  $5 \times 10^{-7} \text{ mol l}^{-1}$  aldosterone (Aldo),  $5 \times 10^{-7} \text{ mol l}^{-1}$  hydrocortisone (HC),  $5 \times 10^{-7} \text{ mol l}^{-1}$  corticosterone (C)] or (ii) aldosterone alone (Aldo:  $5 \times 10^{-7} \text{ mol l}^{-1}$ ). Each of these was used with or without ovine prolactin (PRL,  $2 \mu\text{g ml}^{-1}$ ). Thus, there were four different culture media and these were changed every other day. Prolactin was purchased from Sigma Chemical Co. (St Louis, MO, USA).

### Light microscopy and immunocytochemistry

After the culture period, the skin was fixed with 10% formalin, embedded in paraffin and sectioned at  $8 \mu\text{m}$  thickness. The sections were stained with Haematoxylin 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 using standard methods, as described in our previous paper (Takada *et al.* 1995). In this method, antigen-A-specific antiserum (raised in rabbit; Wako, Oosaka, Japan) diluted 1:10 with phosphate-buffered saline (PBS) is used as the primary antibody and a peroxidase-labelled antibody (raised in goat against rabbit IgG; Sigma) is used as a second antibody, diluted 1:200 with PBS. Finally, peroxidase activity is confirmed cytochemically using the diaminobenzidine (DAB) reaction.

### Measurement of short-circuit current ( $I_{sc}$ )

The dissected or cultured skin samples were mounted in an Ussing-type chamber using silicone gaskets (inner diameter 5 mm) to minimize edge damage. Both sides of the skin were bathed in aerated Ringer's solution containing (in  $\text{mmol l}^{-1}$ ): NaCl, 110; KCl, 2;  $\text{CaCl}_2$ , 1; glucose, 10; Tris, 10; at pH 7.2, and allowed to equilibrate for 1 h. The  $I_{sc}$  was measured under voltage-clamp conditions using a short-circuit current amplifier (CEZ-9100, Nihon Kohden, Tokyo). The fluid resistance was compensated. The resistance across the skin is not mentioned in the text, because the value has already been discussed in our previous papers (Takada, 1985, 1989; Takada *et al.* 1995). When required, amiloride and acetylcholine (ACh) were applied to the apical side of the skin.

### Statistical analyses

Values are expressed as means  $\pm$  s.e.m. Differences were analyzed using one-way analysis of variance (ANOVA) and Scheffé's test for three groups and Student's *t*-test or Welch's test for two groups.

## Results

### Microscopic observations and immunocytochemistry

Fig. 1 shows sections of intact larval skin and cultured skin treated with corticoid either supplemented with PRL or lacking PRL. Larval skin is composed of an outermost layer of apical cells, an innermost layer of basal cells and, between these layers, skein cells (Fig. 1A). After EDTA-treatment to remove apical and skein cells (Takada *et al.* 1995), the skin was cultured with Aldo, Aldo+PRL, corticoid mixture (Mix: Aldo+HC+C) or Mix+PRL for 1–2 weeks. The skin cultured with PRL developed apical cells and seemed to have the characteristics of larval-type skin (Fig. 1B,C). Fig. 1D shows the skin of stage XXV animals. It looks like the adult type in so far as a stratified epidermis is well developed and the outermost layer shows cornification. Fig. 1E,F shows skin cultured with Aldo and with Mix, respectively. The skin seems to be of the adult type since a stratified cell layer is well developed and it is similar in appearance to the skin of stage XXV animals.

Whether skin cultured with corticoid supplemented with PRL is of a larval type cannot be conclusively demonstrated by observation of Haematoxylin–Eosin staining alone. Fortunately, human blood group antigen A is a specific molecular marker for adult-type cells of the bullfrog epidermis, since the epidermis of the adult type, but not that of the larval type, reacts to the antibody (Kaiho and Ishiyama, 1987; Yoshizato *et al.* 1993). For this reason, we used this method to determine whether the skin really was of the adult type (Fig. 2).

As expected, larval skin did not react to the antibody (Fig. 2A). Moreover, the skin cultured with Aldo+PRL or with Mix+PRL did not react to the antibody, suggesting that these samples are of a larval type (Fig. 2B,C). In contrast, the skin from stage XXV animals (Fig. 2D) and the skin cultured with Aldo alone or with Mix (Fig. 2E,F) did react to the antibody, indicating that these skins are of the adult type.

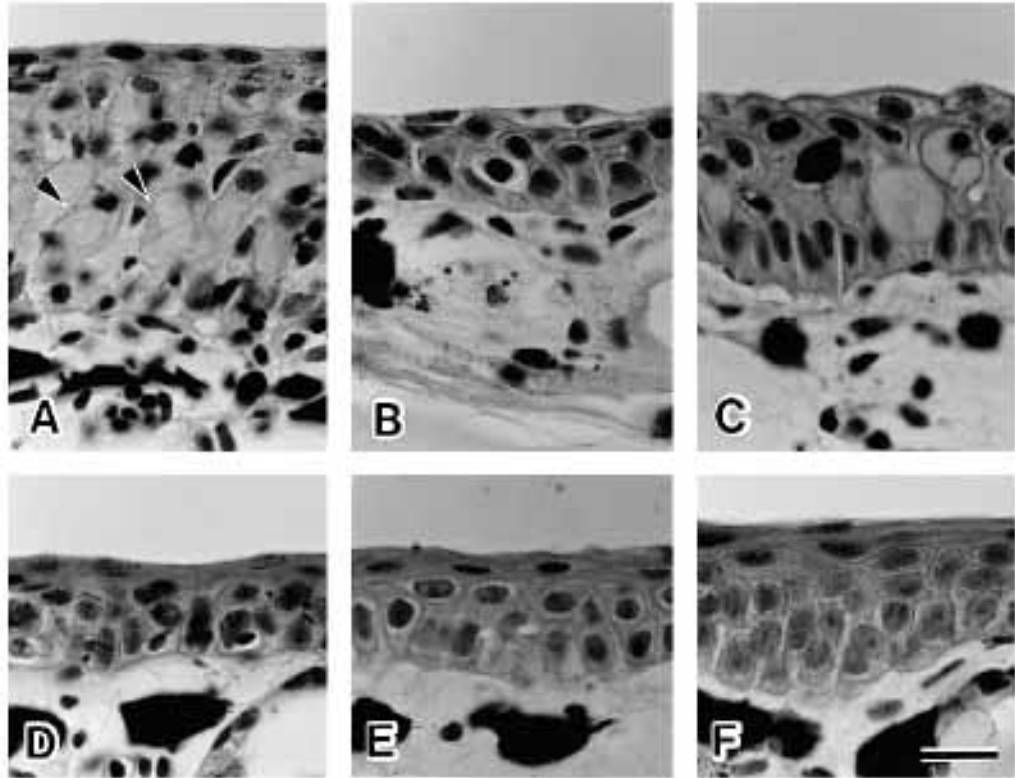


Fig. 1. Haematoxylin and Eosin staining of the skin. (A) Larval skin, (B) skin cultured with aldosterone (Aldo) plus prolactin (PRL), (C) skin cultured with corticoid mixture (Mix) plus PRL, (D) skin at stage XXV, (E) skin cultured with Aldo, (F) skin cultured with Mix. Arrowheads in larval skin (A) indicate typical skin cells. Scale bar, 20  $\mu$ m.

*Effect of acetylcholine and amiloride on  $I_{sc}$*

Amiloride inhibits the  $I_{sc}$  across adult frog skin but acetylcholine (ACh) has no effect on it (Kirschner, 1955; Benos *et al.* 1992). In contrast, ACh and amiloride both stimulate the  $I_{sc}$  in larval skin (Cox, 1993). Thus, an  $I_{sc}$

stimulated by both ACh and amiloride is functional evidence for a skin of the larval type, whereas an amiloride-blockable  $I_{sc}$  on which ACh has no effect is functional evidence for a skin of the adult type. We made use of this distinction in the following experiments.

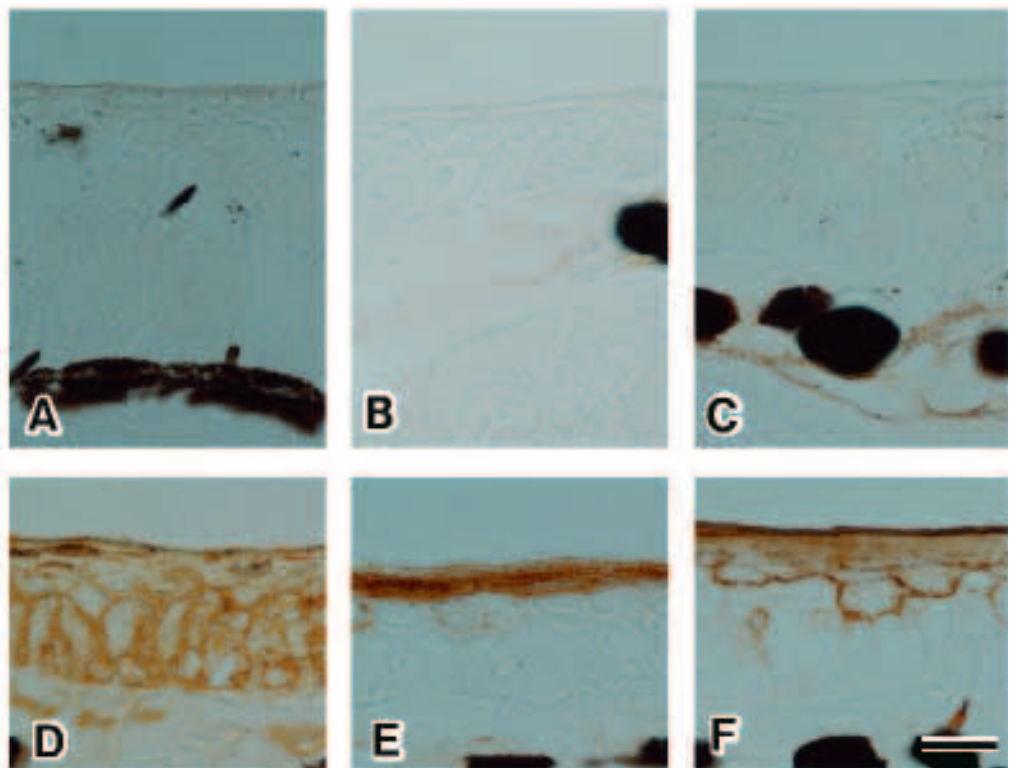


Fig. 2. Immunocytochemical staining of the skin. The skin was stained using antiserum against human blood group antigen A. (A) Larval skin, (B) skin cultured with aldosterone (Aldo) plus prolactin (PRL), (C) skin cultured with corticoid mixture (Mix) plus PRL, (D) skin at stage XXV, (E) skin cultured with Aldo, (F) skin cultured with Mix. Scale bar, 20  $\mu$ m.

Table 1. *Effects of acetylcholine or amiloride on the short-circuit current across the frog skin*

	Larva	Aldo+PRL	Mix+PRL	St XXV	Aldo	Mix
Control value of $I_{sc}$ ( $\mu\text{A cm}^{-2}$ )	0.60±0.19 (24)	0.23±0.06 (20)	0.40±0.12 (31)	25.1±4.5 (10)	11.2±2.0 (18)	12.5±1.5 (23)
$\Delta I_{sc}$ by acetylcholine ( $\mu\text{A cm}^{-2}$ )	0.88±0.23 (13)	1.48±0.32 (10)	1.35±0.24 (24)	-0.56±0.58 (5)	-0.11±0.10 (9)	-0.33±0.11 (7)
$\Delta I_{sc}$ by amiloride ( $\mu\text{A cm}^{-2}$ )	0.56±0.16 (11)	0.59±0.08 (10)	0.69±0.14 (7)	-21.3±7.1 (5)	-9.12±2.12 (9)	-11.5±1.7 (16)

Larva, larval skin; Aldo+PRL, skin cultured with aldosterone (Aldo) plus prolactin (PRL); Mix+PRL, skin cultured with the corticoid mixture (Mix) plus PRL; Aldo, skin cultured with Aldo; Mix, skin cultured with Mix; St XXV, skin at stage XXV (froglet);  $\Delta I_{sc}$ , absolute difference between maximum response and baseline short-circuit current  $I_{sc}$ .

Control values of skin resistance were as follows ( $\text{k}\Omega \text{cm}^2$ ): 0.43±0.03 (larva,  $N=13$ ); 0.44±0.07 (Aldo±PRL,  $N=11$ ); 0.61±0.09 (Mix+PRL,  $N=7$ ); 0.85±0.05 (St XXV,  $N=5$ ); 0.74±0.16 (Aldo,  $N=10$ ); 0.82±0.17 (Mix,  $N=11$ ).

Acetylcholine was added at  $1 \text{ mmol l}^{-1}$ ; amiloride was added at  $10^{-4} \text{ mol l}^{-1}$ .

Values are means  $\pm$  S.E.M. ( $N$ ).

Figs 3 and 4 show typical examples of the effects of ACh and amiloride on  $I_{sc}$ , and the results are summarized in Table 1. Baseline  $I_{sc}$  values in larval skin, in skin cultured with Aldo+PRL and in skin cultured with Mix+PRL are expressed as 'Control value of  $I_{sc}$ '. There are no significant differences between these values (one-way ANOVA,  $F=1.57$ ,  $P>0.2$ ). Although the control value of  $I_{sc}$  was well developed in the skin samples cultured with Aldo or with Mix, both produced significantly lower values than skin from stage XXV animals (Scheffé's test,  $P<0.03$ ). There is no significant difference in terms of  $I_{sc}$  between the skin cultured with Aldo and that cultured with Mix (Scheffé's test,  $P>0.5$ ). However, there are significant differences in terms of baseline  $I_{sc}$  between the larval skin and the skin from stage XXV animals, between the skin cultured with Aldo+PRL and that cultured with Aldo, and between the skin cultured with Mix+PRL and that cultured with Mix (Student's  $t$ -test or Welch's test,  $P<0.001$ ); in each pair, the latter was greater than the former (Table 1).

Apical application of ACh ( $1 \text{ mmol l}^{-1}$ ) induced a transient increase in the  $I_{sc}$  of larval skin, in skin cultured with Aldo+PRL and in skin cultured with Mix+PRL (Fig. 3). There are no significant differences between the evoked increases in the  $I_{sc}$  (absolute difference between values before and after ACh treatment) of these three kinds of skins (one-way ANOVA,  $F=1.13$ ,  $P>0.3$ ; Table 1). Apical application of amiloride ( $10^{-4} \text{ mol l}^{-1}$ ) to these skins also induced a transient increase in  $I_{sc}$  (Fig. 4). Again, there are no significant differences between these three evoked increases in the  $I_{sc}$  (one-way ANOVA,  $F=0.32$ ,  $P>0.7$ ; Table 1).

By contrast, application of ACh had no effect on the  $I_{sc}$  of skin from stage XXV animals, skin cultured with Aldo or skin cultured with Mix (Fig. 3; Table 1). Moreover, application of amiloride ( $10^{-4} \text{ mol l}^{-1}$ ) to these skins induced a decrease in the  $I_{sc}$  in each case (Fig. 4; Table 1). There are no significant differences between the induced decreases in  $I_{sc}$  (absolute difference between values before and after amiloride treatment) of these three kinds of skins (one-way ANOVA,  $F=3.29$ ,  $P>0.05$ ).

The increase in  $I_{sc}$  induced by acetylcholine or amiloride in

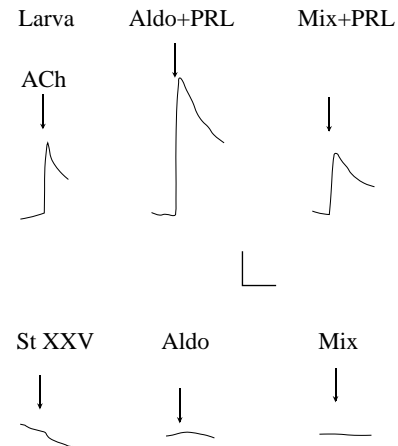


Fig. 3. Typical responses of short-circuit current ( $I_{sc}$ ) to acetylcholine (ACh). Larva, larval skin; Aldo+PRL, skin cultured with aldosterone (Aldo) plus prolactin (PRL); Mix+PRL, skin cultured with corticoid mixture (Mix) plus PRL; St XXV, skin at stage XXV; Aldo, skin cultured with Aldo; Mix, skin cultured with Mix. Arrows indicate application of ACh ( $1 \text{ mmol l}^{-1}$ ). Calibration bars: vertical,  $1 \mu\text{A cm}^{-2}$  (except St XXV,  $10 \mu\text{A cm}^{-2}$ ); horizontal, 2 min.

the skin preparations cultured with Aldo+PRL or with Mix+PRL was similar to that induced in the larval skin, indicating that these cultured skins had the characteristics of a functionally larval type. In contrast, the  $I_{sc}$  of the skins cultured with Aldo alone or with Mix was not influenced by ACh and was actually inhibited by amiloride, as was that of the skin at stage XXV, suggesting that these skins had developed into a functionally adult type.

## Discussion

Corticoids stimulate amiloride-blockable active  $\text{Na}^+$  transport in various organs. In the kidney collecting duct of rat and rabbit, the mineralocorticoid aldosterone and glucocorticoids both increase the  $I_{sc}$  (Náray-Fejes-Tóth and Fejes-Tóth, 1990; Laplace *et al.* 1992). Glucocorticoid does not induce any additional effect on the  $I_{sc}$  over and above the

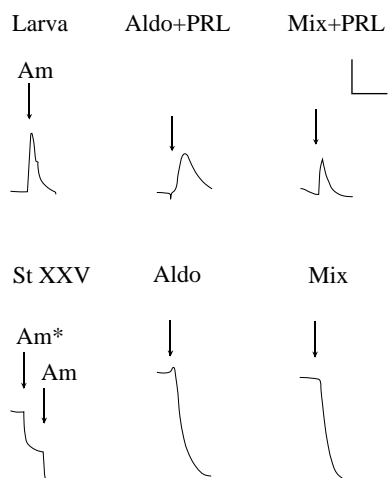


Fig. 4. Typical responses of short-circuit current ( $I_{sc}$ ) to amiloride (Am). Larva, larval skin; Aldo+PRL, skin cultured with aldosterone (Aldo) plus prolactin (PRL); Mix+PRL, skin cultured with corticoid mixture (Mix) plus PRL; St XXV, skin at stage XXV; Aldo, skin cultured with Aldo; Mix, skin cultured with Mix. Arrows indicate application of  $10^{-4} \text{ mol l}^{-1}$  Am or  $10^{-5} \text{ mol l}^{-1}$  Am\*. Calibration bars: vertical,  $1 \mu\text{A cm}^{-2}$  (Larva, Aldo+PRL and Mix+PRL),  $4 \mu\text{A cm}^{-2}$  (Aldo and Mix) or  $20 \mu\text{A cm}^{-2}$  (St XXV); horizontal, 2 min (Larva, Aldo+PRL, Mix+PRL and Mix), 4 min (Aldo) or 8 min (St XXV).

increase induced by aldosterone in the collecting duct of the rat (Laplace *et al.* 1992). By contrast, in the distal or proximal colon of the rat, although aldosterone and glucocorticoid each increased  $I_{sc}$  when given alone, glucocorticoid suppressed the aldosterone-induced increase in the  $I_{sc}$  (Jorkasky *et al.* 1985; Bastl *et al.* 1992). In Amphibia, aldosterone stimulates active  $\text{Na}^+$  transport in A6 cells, toad bladder and frog skin (Palmer and Speez, 1986; Kemendy *et al.* 1992; Verrey, 1995). Glucocorticoid has a greater stimulatory effect on active  $\text{Na}^+$  transport than does aldosterone in the TBM-18-28 cell line from toad bladder, and glucocorticoid intensifies the effect of aldosterone in A6 cells (Watlington *et al.* 1982; Duncan *et al.* 1988; Gaeggeler *et al.* 1993). It is reported that aldosterone stimulates  $\text{Na}^+$  transport *via* glucocorticoid receptors rather than by mineralocorticoid receptors in A6 cells (Schmidt *et al.* 1993). Thus, the actions of mineralocorticoid and glucocorticoid seem to differ among species and among organs.

In the present experiments, EDTA-treated larval skin was cultured with Aldo or with Aldo+HC+C (Mix), each supplemented with PRL or lacking PRL, and the functioning and morphology of these skins were examined. The morphology of the skin cultured with Aldo was similar to that of the skin cultured with Mix; that is, the skin had an adult-type appearance. Such skin preparations not only look like the adult type, but they actually are of the adult type since: (i) the skin preparations reacted to human blood group antigen A, a marker for an adult-type epidermis in bullfrog skin; and (ii) the  $I_{sc}$  of these skin preparations was reduced by amiloride. In addition, there was no functional difference between skin preparations cultured with Aldo or with Mix, since baseline  $I_{sc}$

values and the decrease in  $I_{sc}$  evoked by amiloride were each similar in the two skin preparations (see Results). That is to say, aldosterone acts on basal cells and promotes the development of a morphologically, immunocytochemically and functionally adult-type epidermis; moreover, HC+C neither stimulated nor inhibited the effect of aldosterone.

The concentration of aldosterone used in this experiment ( $5 \times 10^{-7} \text{ mol l}^{-1}$ ) is appropriate for the development of active  $\text{Na}^+$  transport in amphibian epithelium, since the concentration is sufficient to elicit about 90% of the maximum effect of aldosterone on  $I_{sc}$ . No additional action of glucocorticoid was observed on the aldosterone-induced development of the  $I_{sc}$  because mineralocorticoid may affect  $\text{Na}^+$  transport through glucocorticoid receptors, and the concentration of aldosterone may be enough to occupy most of the glucocorticoid receptors (Schmidt *et al.* 1993).

By contrast, the skin cultured with Aldo+PRL or Mix+PRL had not developed adult-type features but, instead, had actually developed into a functionally and immunocytochemically larval type. Moreover, the increases in the  $I_{sc}$  evoked by amiloride and ACh in skin cultured with Aldo+PRL were quite similar to those seen in skin cultured with Mix+PRL (see Results), indicating that HC+C neither stimulated nor inhibited the action of Aldo+PRL.

The apical cells making up the outermost layer of the larval skin seem to be the source of the ACh/amiloride-stimulated  $I_{sc}$ , since such an  $I_{sc}$  was observed in skin cultured with Aldo+PRL, in which apical cells developed, whereas it was never observed in EDTA-treated skin in which only basal cells remained (data not shown).

Aldosterone acts on basal cells and promotes the development of an adult-type epidermis, whereas the action of Aldo+PRL on basal cells is such as to lead to the development of a larval-type epidermis. It has been reported that prolactin enhances the action of corticoid in mammalian mammary glands; that is, PRL enhances the synthesis of casein or  $\alpha$ -lactalbumin induced by cortisol in mouse mammary epithelium or rat mammary tissue (Ray *et al.* 1986; Taketani and Oka, 1986). In Amphibia, PRL is known to antagonize the action of thyroid hormone and to suppress the metamorphic changes, i.e. application of PRL inhibits tail resorption in tadpoles and resorption of tail segments cultured with thyroid hormone (Kikuyama *et al.* 1993). In our experiments, PRL antagonized the action of aldosterone, i.e. PRL inhibited the development of an adult-type epidermis which was otherwise induced by aldosterone.

Prolactin regulates ion transport and osmoregulation in fish (Nishimura, 1985). In Amphibia, PRL has been reported to accelerate the 'second metamorphosis, water drive', causing transformation from the terrestrial to the aquatic phase (White and Nicoll, 1981). In toads, the PRL level is actually high only when individuals stay temporarily in the water during the breeding season (Ishii *et al.* 1989; Yamamoto *et al.* 1989). Stage XXV is the final stage of the metamorphosis of the bullfrog tadpole. By then, its characteristics have changed from those suitable for the aquatic to those suitable for the terrestrial environment and amiloride-blockable active  $\text{Na}^+$  transport has

already differentiated. An application of PRL for 2 weeks to animals at this stage induces a decrease in the  $I_{sc}$  (Takada, 1989). Whether the decrease in the  $I_{sc}$  induced by PRL treatment is relevant to the change from the terrestrial to the aquatic environment is not yet known.

Acetylcholine stimulation of the  $I_{sc}$  in the larval skin of the bullfrog is exerted *via* nonselective cation channels typical of larval skin (Cox, 1993), whereas the amiloride-induced decrease in  $I_{sc}$  is exerted *via* amiloride-blockable  $Na^+$  channels typical of adult skin (Benos *et al.* 1992). The roles of ACh-stimulated nonselective cation channels in larval skin and of the amiloride-blockable  $Na^+$  channels that develop in the skin at the climax stages of metamorphosis and which adapt the animal to the terrestrial rather than to the aquatic environment remain to be elucidated.

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