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[Short Communication]

In vivo Treatment of Bullfrog Tadpoles with Aldosterone Potentiates ACh-Receptor Channels, but not Amiloride-Blockable Na^+ Channels in the Skin

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ABSTRACT—Amiloride-blockable Na^+ channels participate in active Na^+ transport across adult, but not larval, bullfrog skin. Their development is induced *in vitro* by culturing the tadpole skin with aldosterone. When tadpoles were raised in aldosterone (5×10^{-7} M) for 2 weeks, however, neither development of such channels nor localization of antigen A, a marker of adult-type epidermis, was seen, the skin still being of the larval type. In contrast, aldosterone treatment did potentiate (by a factor of two) the activity of the acetylcholine receptor (ACh-receptor) channel, a functional marker of larval-type skin. The short-circuit current (SCC) across the skin, far from being inhibited by amiloride, was stimulated by both amiloride and ACh. The nystatin-stimulated SCC was about twice its control amplitude, suggesting that the aldosterone treatment also potentiated the activity of the Na^+ pump.

INTRODUCTION

Larval-type bullfrog skin possesses acetylcholine (ACh)-receptor channels, stimulated by both ACh and amiloride (Cox, 1992, 1993; Takada *et al.*, 1996a, b). The amiloride-blockable active Na^+ transport, a feature of adult skin, develops at stages XXI-XXII of the climax stage of metamorphosis and increases thereafter (Cox and Alvarado, 1979, 1983; Hillyard *et al.*, 1982; Takada, 1985). Recently, Takada *et al.* (1995a) found that the amiloride-blockable Na^+ channel develops when tadpole skin was cultured with aldosterone. This was unexpected because the development of such active Na^+ transport had been assumed to require thyroid hormones (Cox and Alvarado, 1979; Takada, 1985).

The present study was undertaken to examine whether aldosterone stimulates the development of amiloride-blockable active Na^+ transport also in the *in vivo* situation.

MATERIALS AND METHODS

Animals

Tadpoles of *Rana catesbeiana* at stages XI-XVI were purchased from a local animal supplier in Misato City (Saitama, Japan). The stages were determined according to Taylor and Kollros (1946). They were maintained in tap water (control) or in a 5×10^{-7} M solution of aldosterone in tap water (aldosterone-treated), and fed with boiled spinach. The hormone concentration was selected as it induced adequate increase in amiloride-blockable SCC in *in vitro* experiment (Takada *et al.*, 1995a). After 2 weeks, none of the tadpoles had reached stage XX. They were then anesthetized with iced water, and portions of dorsal body skin dissected out.

Light microscopy and immunocytochemistry

Skin fixed with 10% formalin and embedded in paraffin was sectioned at 8 μm thickness. Some sections were stained with hematoxylin and eosin. Sections for immunocytochemistry were prepared similarly, and the localization of antigen A was detected as described previously (Takada *et al.*, 1995a). In brief, antigen A-specific antiserum (raised in rabbit) diluted 1:10 with phosphate-buffered saline (PBS) was used as primary antibody. As a second antibody, peroxidase-labeled antibody (raised in goat against rabbit immunoglobulin G) diluted 1:200 with PBS was used. Finally, peroxidase activity was confirmed cytochemically using the diaminobenzidine reaction.

Measurement of short-circuit current (SCC)

The dissected portion of skin was mounted in a Ussing-type chamber with silicone gaskets (inner diameter 5 mm) to minimize edge damage. Both sides of the skin were bathed in aerated Ringer's solution containing (mM): NaCl, 110; KCl, 2; $CaCl_2$, 1; glucose, 10; Tris, 10; at pH 7.2. After a 1 hr period for equilibration, the potential difference (PD) was measured under open-circuit conditions, then clamped to zero using a short-circuit current amplifier (CEZ-9100, Nihon Kohden, Tokyo). The fluid resistance was compensated. The effects of ACh (1 mM), amiloride (10^{-4} M), nystatin (50 μg/ml), and ouabain (10^{-5} M) on SCC were measured under voltage-clamp conditions. Amiloride, ACh, and nystatin were applied to the apical side, and ouabain to the basolateral side. The effect of each chemical is presented as the difference between the SCC value obtained before its application and the peak SCC value after its application. The peak response to each chemical occurred as follows: amiloride and ACh, within 4

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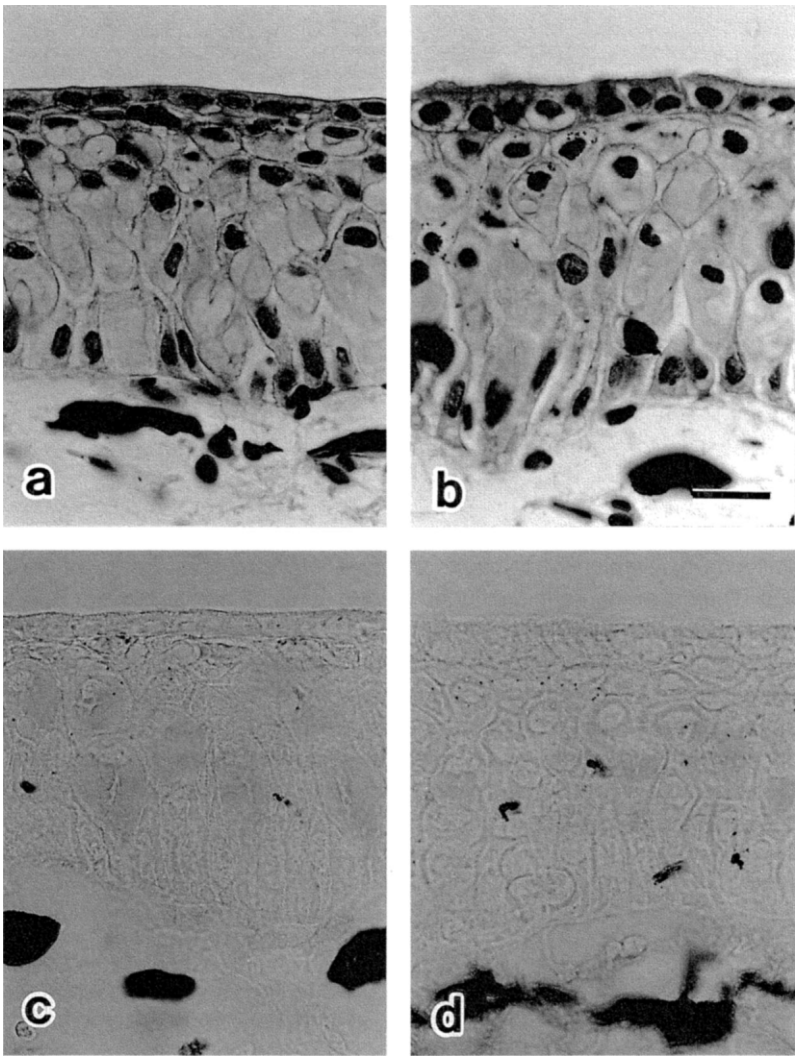

Fig. 1. Hematoxylin and eosin staining (**a**, **b**), and immunocytochemical staining (**c**, **d**) of the skin. (**a**, **c**) tadpoles raised in tap water ("control"); (**b**, **d**) tadpoles raised in aldosterone (5×10^{-7} M) for 2 weeks. Bar: 20 μm.

min; nystatin, within 8 min; ouabain, within 30-40 min.

Statistical analysis

Differences were analyzed using a Student's t-test and taken as significant when $P<0.05$.

RESULTS AND DISCUSSION

Larval epidermis of the bullfrog is composed of apical cells in the outermost layer, basal cells in the innermost layer, and skein cells in the intermediate layer (Robinson and Heintzelman, 1987). In the present study, the skin samples from tadpoles raised in tap water (control) or in aldosterone solution were virtually indistinguishable from each other; that is, the epidermis of the aldosterone-treated tadpole was morphologically of the larval type (Fig. 1a, b). Human blood group antigen A is a specific molecular marker for the adult-type epidermis of bullfrog skin (Kaiho and Ishiyama, 1987; Takada *et al.*, 1995a, 1996a; Yoshizato *et al.*, 1993). When the skin of aldosterone-treated tadpoles was exposed to antigen A specific antiserum, no cross reaction was seen, indicating that the skin was of the larval type (Fig. 1c, d).

In the next experiment, we examined the effects of ACh and amiloride on the SCC across the skin. In tadpole skin, application of ACh or amiloride induces a transient increase in SCC (Cox, 1992, 1993), whereas amiloride causes a decrease in SCC in adult skin. Amiloride-blockable Na^+ channels are known to develop at stages XXI-XXII of metamorphosis, and thereafter, the SCC is reduced by amiloride (Cox and Alvarado, 1979; Hillyard *et al.*, 1982; Takada, 1985).

Table 1 shows PD, SCC, and resistance (R) measurements. The SCC was almost 7 times greater in aldosterone-treated tadpoles than in the controls ($p<0.001$). The development of a higher SCC during metamorphosis had been assumed to indicate the development of amiloride-blockable Na^+ channels (Cox and Alvarado, 1979; Takada, 1985). As shown in Fig. 2 and Table 2, however, both ACh and amiloride in-

Table 1. PD, SCC, and R of skin of *R. catesbeiana*

	PD (mV)	SCC ($\mu A/cm^2$)	R ($k\Omega \cdot cm^2$)
Control (n=24)	0.19 ± 0.05	0.54 ± 0.19	0.34 ± 0.03
Aldo (n=36)	3.17 ± 0.34	3.70 ± 0.39	0.82 ± 0.04

Control: Animals raised in tap water. Aldo: Animals raised in aldosterone (5×10^{-7} M) for 2 weeks. All values (PD, SCC, and R) under Aldo are significantly different from Control ($p<0.001$ in each case).

creased the SCC in aldosterone-treated tadpoles. The ACh-induced SCC increase in aldosterone-treated tadpoles was larger by a factor of two than in control ($p<0.01$), although no significant effect of amiloride was seen ($p>0.1$). The absence of an inhibitory effect of amiloride indicates that amiloride-blockable Na^+ channels were not induced with aldosterone. Instead, the stimulative effect of ACh suggests that ACh-receptor channels was activated by the aldosterone-treatment.

Activation of ACh-receptor channels is unlikely to be the only cause for the higher baseline SCC in aldosterone-treated tadpoles. Stimulation of the Na^+ pump (Na,K-ATPase) would also cause an increase in the SCC. Aldosterone is known to potentiate the Na^+ pump in A6 cells, in rabbit CCD cells, and in toad bladder (Benos *et al.*, 1992; Verrey, 1995). If Na^+ pump activity is stimulated by aldosterone, the increase in SCC evoked by the apical application of nystatin should be larger in aldosterone-treated tadpoles than in control, because nystatin increases the cation permeability of the apical membrane (Cox and Alvarado, 1983). As shown in Fig. 2 and Table 2, the nystatin-induced increase in SCC was greater by a factor of two in aldosterone-treated tadpoles than in the controls ($p<0.05$). In addition, the SCC of aldosterone-treated tadpoles was decreased by ouabain (Table 2). These results suggest that the higher SCC of aldosterone-treated skin is due to potentiation not only of ACh-receptor channels but also of the Na^+ pump.

Although amiloride-blockable Na^+ channels can be developed in the tadpole skin with aldosterone alone *in vitro* (Takada *et al.*, 1995a, 1996a), these channels were not induced in the present study in tadpoles before stage XX under *in vivo* condition. We have also shown that prolactin antagonizes the aldosterone's action in vitro (Takada *et al.*, 1995b). In the present study, however, no attempt was made to correlate the effect of aldosterone to prolactin cell activity. Further studies are certainly called for to clarify the discrepancy between the *in vitro* and *in vivo* effects of aldosterone, including interaction with not only prolactin but also other hormones such as thyroid hormones, growth hormone, IGFs and EGFs, etc.

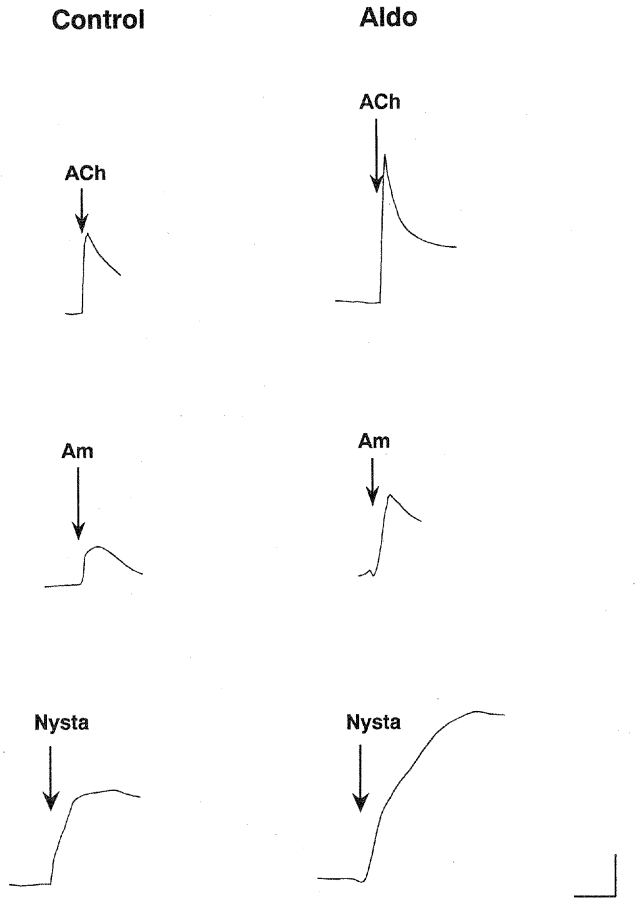

Fig. 2. Typical examples of effect of acetylcholine, amiloride, and nystatin on SCC of the skin. (**Control**) tadpoles raised in tap water; (**Aldo**) tadpoles raised in aldosterone (5×10^{-7} M) for 2 weeks. ACh: 1 mM acetylcholine. Am: 10^{-4} M amiloride. Nysta: 50 μg/ml nystatin. Vertical: 1 μA (except nystatin under Aldo: 2 μA). Horizontal: 2 min.

Table 2. Effects of ACh, amiloride, ouabain, and nystatin on ΔSCC ($\mu A/cm^2$).

	ACh	Am	Ouabain	Nystatin
Control	1.04 ± 0.24 (n=11)	0.65 ± 0.18 (n=8)	—	4.21 ± 1.36 (n=6)
Aldo	2.36 ± 0.36* (n=20)	1.10 ± 0.26 (n=11)	−3.07 ± 0.77 (n=8)	9.85 ± 1.53** (n=5)

ΔSCC: change in SCC (peak value) on application of ACh, Am, ouabain, or nystatin. ACh: acetylcholine (10^{-3} M). Am: amiloride (10^{-4} M). Ouabain: 10^{-5} M. Nystatin: 50 μg/ml. Control: animals raised in tap water. Aldo: animals raised in aldosterone (5×10^{-7} M) for 2 weeks. —: value not determined. Aldosterone significantly increased the ACh and nystatin effects, but not the amiloride effect (*, $p<0.01$; **, $p<0.05$).

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