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Growth hormone is a weaker candidate than prolactin for the hormone responsible for the development of a larval-type feature in cultured bullfrog skin

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Summary

Prolactin (PRL) has, for some years, been considered to be the 'juvenile hormone' in amphibians. Recently, growth hormone (GH) has been proposed as another candidate, because in the larval stages the expression of the mRNA GH is high but it is downregulated in the climax stages of metamorphosis or following treatment with thyroid hormone. In the present study, we investigated whether GH promotes the development of one particular larval-type feature of bullfrog tadpole skin *in vitro*. The amiloride-, acetylcholine- and ATP-stimulated short-circuit current (SCC) is a physiological marker of larval-type bullfrog skin. These types of ligand-stimulated SCC (1) developed when EDTA-treated tadpole skin was cultured with corticoids supplemented with PRL or GH and (2) were not significantly different between skin cultured with PRL and intact tadpole skin. However, the amiloride-induced SCC response in skin cultured with GH differed in its kinetics from that of the intact (control) tadpole. On this basis, PRL seems a better candidate than GH for the juvenile hormone, at least with regard to the development of amiloride-stimulated non-selective cation channels.

Key words: growth hormone, prolactin, amphibian metamorphosis, non-selective cation channel, bullfrog skin, *Rana catesbeiana*.

Introduction

In the bullfrog tadpole, the short-circuit current (SCC) across the skin is small and is stimulated by amiloride, acetylcholine and ATP. This is due to stimulation by these ligands of the non-selective cation channel present in such skin. In fact, the amiloride-, acetylcholine- and ATP-stimulated SCC is considered to be a physiological marker of larval-type features in the skin of the bullfrog tadpole (Hillyard et al., 1982a,b; Hillyard and Van Driessche, 1989; Cox, 1992, 1993, 1997).

During the climax stages of metamorphosis, these ligand-stimulated SCCs disappear, while active Na^+ transport, measured as an amiloride-SCC (ASCC) across the skin, develops (Cox and Alvarado, 1979; Hillyard et al., 1982a; Takada, 1985). The development of this ASCC is due to the development of the epithelial Na^+ channel (ENaC). The presence of ASCC and ENaC is a marker for adult-type features in the skin.

Although the development of adult-type features is induced by thyroid hormone *in vivo* (for reviews, see Dodd and Dodd, 1976; Kikuyama et al., 1993), the ASCC is not developed by thyroid hormone *in vitro* but by corticoids (Takada et al., 1995a). This contrast between the *in vitro* and *in vivo* situations is an important issue in the study of amphibian metamorphosis (Takada et al., 1999).

Whereas the development of adult-type features is induced by thyroid hormone or corticoids, prolactin (PRL) is considered to maintain larval-type features in amphibians since the regression of the isolated tail seen under treatment with thyroid hormone is inhibited by PRL (for reviews, see Dodd and Dodd, 1976; Kikuyama et al., 1993). If PRL is indeed the 'juvenile hormone', the serum concentration of PRL would be expected to be higher in the larval stages than in the climax stages of metamorphosis. However, it is actually lower in the larval stages and increases during the climax stages of metamorphosis (Yamamoto and Kikuyama, 1982). In addition, the mRNA for the PRL receptor increases during metamorphosis (Yamamoto et al., 2000). On this basis, PRL seems unlikely to be the juvenile hormone, and other hormone(s) have been proposed for this role (Huang and Brown, 2000a,b; Yamamoto et al., 2000).

The expression of the mRNA for growth hormone (GH) is higher in the larval stages in *Xenopus laevis* and is downregulated in the climax stages of metamorphosis or following treatment with thyroid hormone (Buckbinder and Brown, 1993). Therefore, GH may be the crucial juvenile hormone in amphibians. In fact, overexpression of GH has been shown to stimulate growth in the tadpoles of *X. laevis* (Huang and Brown, 2000a).

To facilitate investigations of the development of larval-type and adult-type features in bullfrog skin, Takada et al. (1995a) developed a method for culturing larval skin. The epidermis of the skin of the larval bullfrog is composed of three types of cells: apical, skein and basal cells (Robinson

and Heintzelman, 1987). EDTA treatment removes both apical and skein cells, leaving only basal cells, and the amiloride- and acetylcholine-responses are not present in such EDTA-treated skin (Takada et al., 1995a,b, 1996). EDTA-treated skin cultured with corticoids develops an adult-type feature: a large SCC that is blocked by amiloride. By contrast, skin cultured with corticoids supplemented with prolactin (PRL) develops a larval-type feature: a small SCC that is stimulated by amiloride and acetylcholine (Takada et al., 1996). However, whether GH also induces the development of amiloride-, acetylcholine- or ATP-stimulated SCC has not been investigated.

Here, we report that these types of ligand-stimulated SCC develop when EDTA-treated larval bullfrog skin is cultured with corticoids supplemented with GH or PRL. However, contrary to our expectations, some of the characteristics shown by the ligand-stimulated SCCs differed between skin cultured with corticoids supplemented with GH and intact tadpole skin.

Materials and methods

Culture of skin and measurement of SCC

EDTA-treated ventral skin from *Rana catesbeiana* (Shaw) tadpoles at stages XII–XV was used, the stages being determined by reference to the work of Taylor and Kollros (1946). Details of the method used for the dissection and culture of the skin have been published elsewhere (Takada et al., 1995a). With the skin mounted in an Ussing-type chamber, the short-circuit current (SCC) was measured under voltage-clamp conditions. The skin resistance (*R*) was calculated as previously described (Takada et al., 1995a). Amiloride (10^{-4} mol l^{-1}), acetylcholine (10^{-3} mol l^{-1}) and ATP (10^{-3} mol l^{-1}) was applied to the apical side of the skin. We chose these concentrations because they induce sizeable SCC responses that, in the case of amiloride, first involve activation and then involve blockade (Hillyard and Van Driessche, 1989; Cox, 1992, 1993, 1997). The change in SCC (ΔSCC), as induced by ligands, was measured as the difference between the peak response and the baseline SCC.

Solutions

RPMI solution, prepared as follows, was used as the culture medium. RPMI 1640 (GIBCO, Grand Island, NY, USA) was diluted to 70% with distilled water and supplemented with corticoids (10^{-6} mol l^{-1} aldosterone, 5×10^{-7} mol l^{-1} each of hydrocortisone and corticosterone), 16.6 mmol l^{-1} $NaHCO_3$, 10 mmol l^{-1} Hepes (pH 7.4), 100 i.u. ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. The skin was cultured in the above medium with or without prolactin (PRL), growth hormone (GH) or T_3 (3,3′,5-triiodo-L-thyronine). The composition of the Ringer's solution used for the measurement of the SCC was 110 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 1 mmol l^{-1} $CaCl_2$, 10 mmol l^{-1} glucose and 10 mmol l^{-1} Tris at pH 7.2. Ovine PRL and porcine GH, T_3, ATP and amiloride were purchased from Sigma Chemicals (St Louis, MO, USA).

Statistical analyses

Statistical significance was assessed using a one-way analysis of variance (ANOVA) followed by Scheffé's test (for three groups), Student's *t*-test or Welch's test (for two groups).

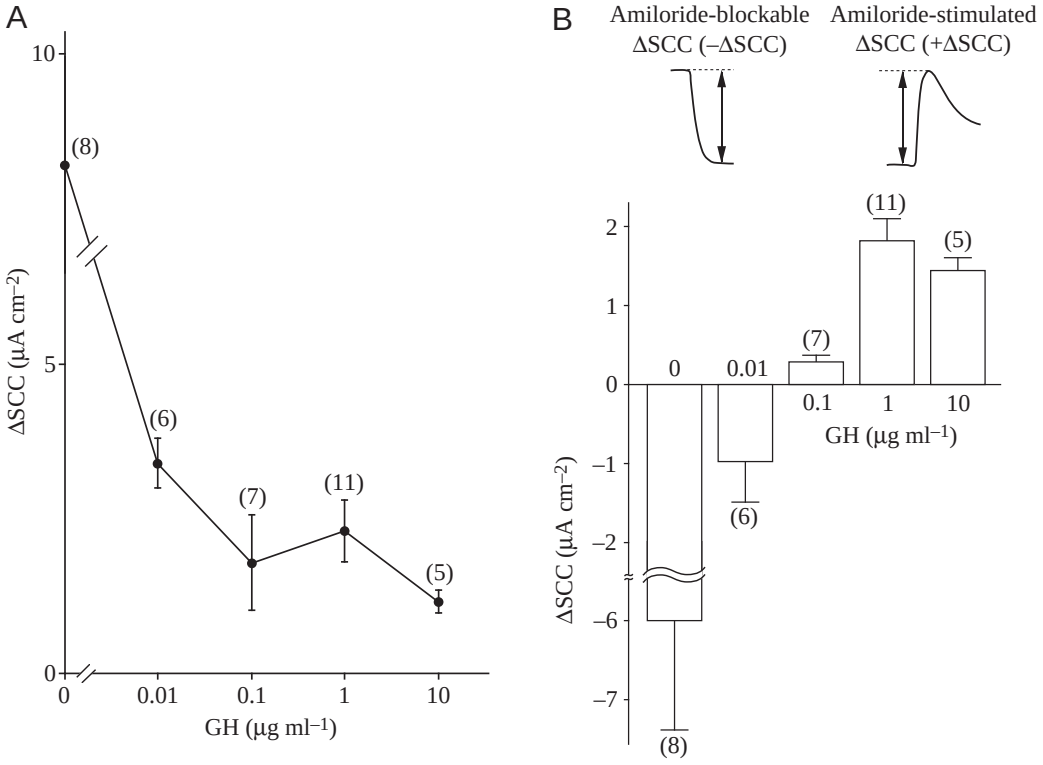

Fig. 1. Effect of growth hormone (GH) on development of short-circuit current (SCC) across cultured tadpole skin (A) and effect of amiloride (Am) on the SCC (B). Skin of tadpoles at stages XII–XV was cultured for 11–13 days with corticoids supplemented with various concentrations of GH. Amiloride (10^{-4} mol l^{-1}) was applied to the apical side of the skin. ΔSCC was measured as the difference between the peak response and the baseline SCC, as indicated in B. Values are means ± S.E.M. (no. of experiments is indicated in parentheses).

Results

EDTA-treated ventral skin was used as the basic material for culture. Fig. 1 shows the SCC recorded from EDTA-treated tadpole skin cultured for 11–13 days with corticoids supplemented with GH. GH clearly inhibited the development of this SCC. If larval-type features do indeed develop in skin cultured with GH, then an amiloride-stimulated SCC should develop. In fact, an amiloride-stimulated SCC did develop when this skin was cultured in the presence of >0.1 μg ml^{-1} GH (Fig. 1B), suggesting that the skin had developed at least this larval-type feature. To determine whether the characteristics of this feature developed in skin cultured with GH are similar to those shown by intact (control) tadpole skin, the effects of amiloride, acetylcholine and ATP on the SCC were investigated.

Fig. 2 shows typical examples and Fig. 3 a summary of the results. EDTA-treated tadpole skin was cultured with corticoids supplemented with PRL (1 μg ml^{-1}) or GH (1 μg ml^{-1}), since this concentration of PRL or GH is sufficient for the development of larval-type features (Takada et al., 1995b; Fig. 1B). In this experiment, the SCC and skin resistance (*R*) of intact tadpole skin were 2.7±0.4 μA cm^{-2} and 3.0±0.3 kΩ cm^{-2}, respectively (*N*=26). Amiloride, acetylcholine and ATP all induced a transient increase in SCC in intact (control) tadpole skin; i.e. the SCC increased rapidly then declined, as other investigators have shown (Hillyard and Van Driessche, 1989; Cox, 1992, 1993, 1997; Takada et al., 1996; Fig. 2).

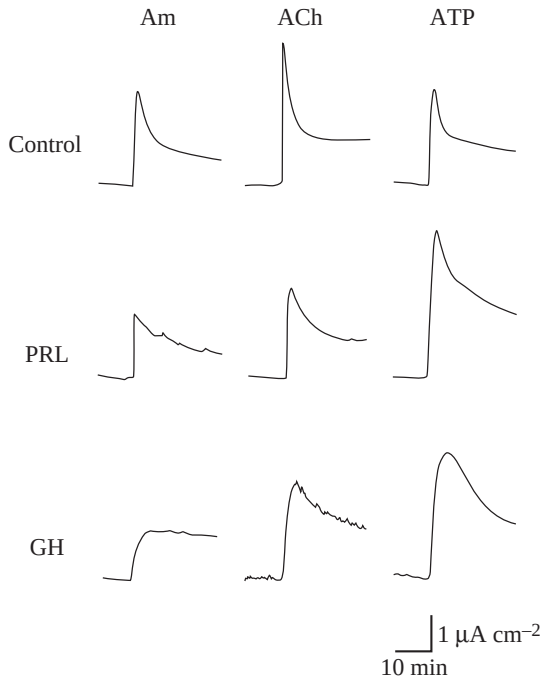

Fig. 2. Typical examples of effects of amiloride (Am; 10^{-4} mol l^{-1}), acetylcholine (ACh; 10^{-3} mol l^{-1}) and ATP (10^{-3} mol l^{-1}) on short-circuit current (SCC) of tadpole skin cultured for 11–13 days with corticoids supplemented with growth hormone (GH; 1 μg ml^{-1}) or prolactin (PRL; 1 μg ml^{-1}), with intact tadpole skin included as control.

The SCC and *R* recorded from skin cultured with corticoids supplemented with PRL were 2.1±0.5 μA cm^{-2} and 2.4±0.4 kΩ cm^{-2}, respectively (*N*=18). Three characteristics of the responses induced by amiloride, acetylcholine and ATP (the ΔSCC, the time from the onset of the response to its peak and the percentage of the normalized peak ΔSCC remaining at 20 min after peak) were not significantly different between skin cultured with PRL and intact tadpole skin (Fig. 3A–C).

The SCC and *R* recorded from skin cultured with corticoids supplemented with GH were 2.6±0.5 μA cm^{-2} and 2.7±0.3 kΩ cm^{-2}, respectively (*N*=22). The acetylcholine- and ATP-induced responses were not significantly different from those seen in intact tadpole skin (Figs 2, 3). However, the amiloride-induced response did differ from that seen in intact tadpole skin insofar as both the time from onset to peak and the relaxation kinetics were significantly slower in skin cultured with GH than in intact tadpole skin (Fig. 3B,C).

Thyroid hormone indirectly inhibits the action of GH in vivo, since it downregulates the expression of the mRNA for GH in *X. laevis* (Buckbinder and Brown, 1993). Whether thyroid hormone directly inhibits the action of GH on the development of a larval-type feature was investigated *in vitro* in the present study. To this end, EDTA-treated tadpole skin was cultured with corticoids supplemented with GH plus T_3 (Fig. 4). The SCC and *R* recorded from skin cultured with corticoids supplemented with GH plus T_3 were 2.3±0.5 μA cm^{-2} and 1.9±0.4 kΩ cm^{-2}, respectively (*N*=16). Thyroid hormone did not inhibit the development of the amiloride-, acetylcholine- and ATP-induced responses in skin cultured with GH (i.e. there are no *P*-values below 0.06 in Fig. 4).

Discussion

The P2X-receptor family comprises a group of non-selective cation channels, all of which are activated by ATP. Recently, Jensik et al. (2001) cloned an ATP-sensitive P2X receptor from bullfrog tadpole skin and named it $P2X_8$. When they injected the cDNA into the oocyte of *X. laevis*, the oocyte responded to ATP but not to amiloride or acetylcholine, suggesting that the ATP-responsive channel and the amiloride- or acetylcholine-responsive channel(s) are distinct.

The same year, Khakh et al. (2001) succeeded in expressing $P2X_2$ or $P2X_3$ receptors from hippocampal neurons on *Xenopus* oocytes. The $P2X_2$ receptors opened rapidly and remained open as long as ATP was present, whereas the $P2X_3$ receptor opened rapidly but then desensitized rapidly. Following co-expression of the $P2X_2$/$P2X_3$ receptors, the response to ATP was almost the same as the sum of the responses seen when $P2X_2$ and $P2X_3$ receptors were expressed separately; i.e. rapid opening then desensitization, with some current remaining as long as ATP was present.

In the present study, the ΔSCC and desensitization kinetics seen in the response to ATP were similar between intact

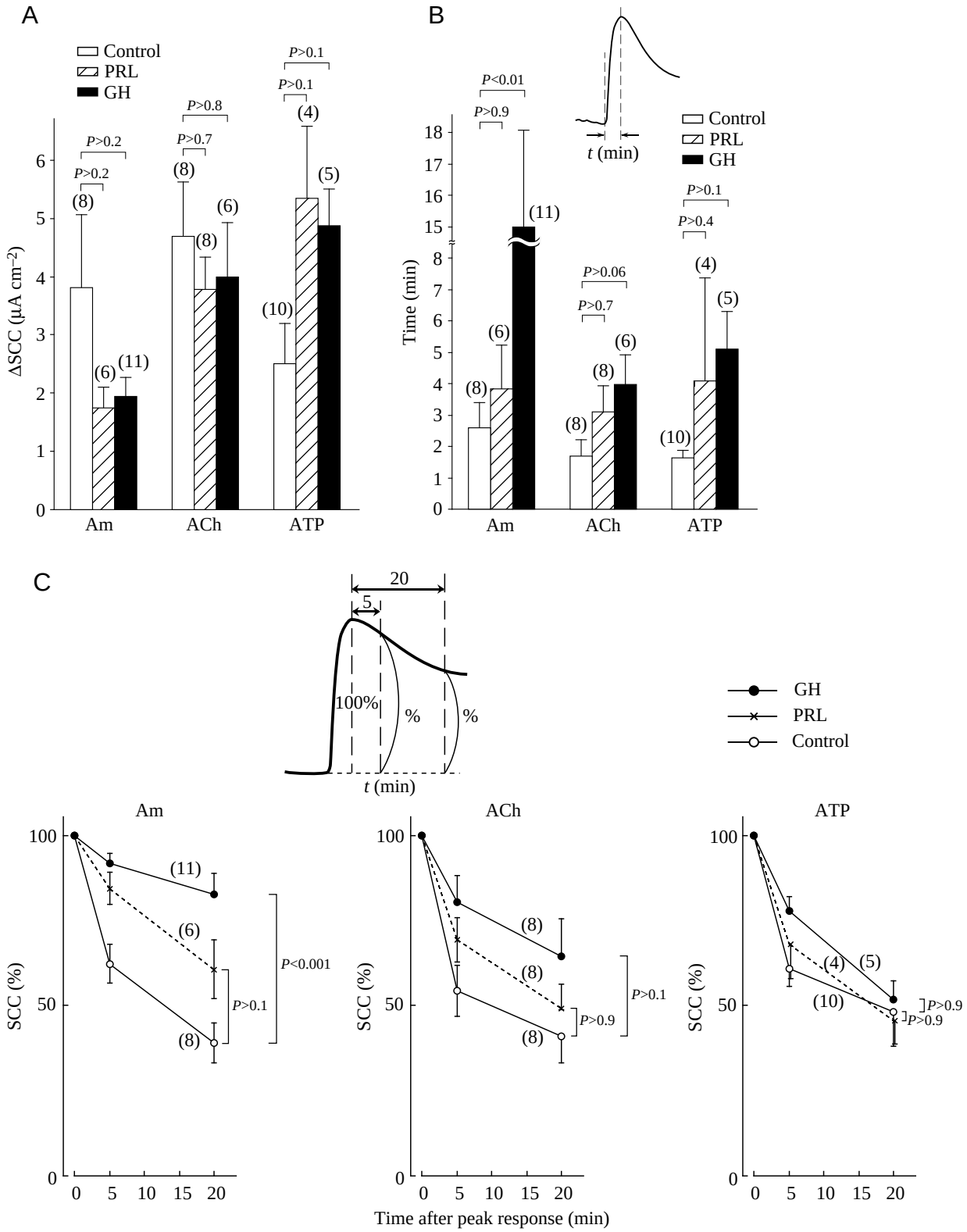

Fig. 3. Summary of effects of amiloride (Am; 10^{-4} mol l^{-1}), acetylcholine (ACh; 10^{-3} mol l^{-1}) and ATP (10^{-3} mol l^{-1}) on short-circuit current (SCC) of tadpole skin cultured for 11–13 days with growth hormone (GH; 1 μg ml^{-1}) or prolactin (PRL; 1 μg ml^{-1}), with intact tadpole skin included as control. (A) ΔSCC was measured as the difference between the peak response and the baseline SCC. (B) Time (*t*) from onset of increase in SCC to peak SCC, measured as indicated. (C) Normalized SCC (%) at 5 min and 20 min after peak response. The SCC measured at 5 min or 20 min after peak was normalized with respect to the maximum response (the difference between peak and baseline SCC being taken as 100%). Values are means ± S.E.M. (no. of experiments is indicated in parentheses).

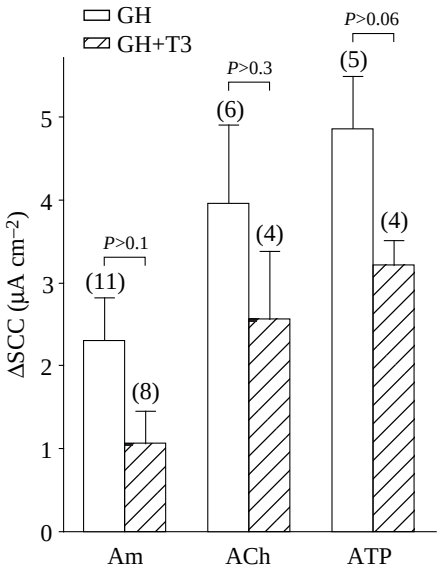

Fig. 4. Effects of amiloride (Am; 10^{-4} mol l^{-1}), acetylcholine (ACh; 10^{-3} mol l^{-1}) and ATP (10^{-3} mol l^{-1}) on tadpole skin cultured with corticoids supplemented with growth hormone (GH; 1 μg ml^{-1}) plus T_3 (10^{-8} mol l^{-1}). Open bars indicates tadpole skin cultured for 11–13 days with corticoids supplemented with GH (these values are also shown in Fig. 3A and are included here to facilitate comparison). Hatched bars indicate tadpole skin cultured for 11–13 days with corticoids supplemented with GH plus T_3. Values are means ± S.E.M. (no. of experiments is indicated in parentheses).

(control) tadpole skin and skin cultured with PRL, and between intact tadpole skin and skin cultured with GH (Figs 2, 3). Hence, similar ATP-sensitive non-selective cation channel(s) may be developed in these skins. It is possible that two kinds of channels destined to mediate the ATP response develop in these skins, since desensitization was slow and the response resembled that exhibited by an oocyte in which $P2X_2/P2X_3$ receptors were co-expressed (see fig. 1 in Khakh et al., 2001).

In our experiment, the acetylcholine-induced response was similar between intact (control) tadpole skin and skin cultured with PRL, and between intact tadpole skin and skin cultured with GH (Figs 2, 3). Hence, similar acetylcholine-responsive channels may be developed in these skins, although whether there are two kinds of acetylcholine-responsive channels, or just one, is unknown as yet.

Hillyard and Van Driessche (1989) have suggested that (1) there are two kinds of binding sites for amiloride, a high-affinity site for activation and a low-affinity site for inhibition or (2) at least two kinds of NSCCs (nonselective cation channels) mediate the amiloride response, one for activation and the other for inhibition, since a low concentration of amiloride (<50–100 μmol l^{-1}) simply stimulates, and does not inhibit, the SCC across larval bullfrog skin, whereas higher concentrations (>100 μmol l^{-1}) first stimulate then block the SCC. Recently, Takada et al. (2001) showed that amiloride simply blocked, and did not stimulate, the SCC across larval bullfrog skin in the presence of Cu^{2+}, even when the concentration of amiloride was low (10^{-6} mol l^{-1}), suggesting that the high-affinity site for amiloride, or the NSCC that is stimulated by amiloride, is blocked by Cu^{2+}.

The amiloride-induced response seen in skin cultured with GH differed in its kinetics from that seen in intact (control) tadpole skin (Figs 2, 3). Growth hormone thus apparently induced the development of a different amiloride-responsive channel than that developed in intact tadpole skin. The present results seem to show mainly activation by amiloride in skin cultured with GH. However, it is possible that such skin does contain some amiloride-blockable (low-affinity) NSCC or some low-affinity (amiloride-blockable) binding sites (mediating inhibition). To explain these possibilities will require further experiments comparing amiloride responses in the presence and absence of Cu^{2+} (which blocks stimulation by amiloride; see above).

Previously, our group showed that adult-type features are developed by bullfrog skin *in vitro* under the influence of aldosterone but not thyroid hormone (Takada et al., 1995a). However, *in vivo*, such features are developed with thyroid hormone but not with aldosterone (Takada et al., 1997, 1999). We therefore hypothesized a few years ago that aldosterone is the crucial hormone for the development of adult-type features but that the effect of aldosterone is suppressed by some means until the suppression is removed by thyroid hormone during the climax stages of metamorphosis, so allowing the development of adult-type features to proceed (Takada et al., 1999). We now feel that growth hormone is an attractive candidate for a factor participating in the suppression of the aldosterone-induced development of adult-type features, since (1) the expression of the mRNA for GH is higher in the larval stages, (2) this expression is inhibited by thyroid hormone during the climax stages of metamorphosis and (3) GH inhibits the development of at least one adult-type feature by corticoids (Buckbinder and Brown, 1993; Fig. 1). However, PRL seems a stronger candidate than GH for the role of juvenile hormone since the characteristics of the amiloride-responsive channel developed in the presence of PRL were closer to those of the channel found in intact tadpole skin than those of the channel developed in the presence of GH. How PRL and/or GH might act as juvenile hormone(s) in amphibians is a matter for future consideration.

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