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Prolactin increases open-channel density of epithelial Na^+ channel in adult frog skin

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Summary

The short-term effect of prolactin on the skin of the adult tree frog *Hyla arborea japonica* was investigated using current-fluctuation analysis. Basolateral application of ovine prolactin (10 μg ml^{-1}) (1) increased the amiloride-blockable short-circuit current (SCC) across the skin 2.6±0.4-fold and (2) increased the open-channel density (*M*) of the epithelial Na^+ channel 6.1±1.2-fold but decreased the single-channel current i to 0.4±0.1 times the control value (*N*=9). The increase in SCC induced by prolactin was thus due to an increase in *M*, not i. Apparently, in amphibians prolactin has not only a counteracting effect on metamorphosis but also a stimulatory effect on the development of adult-type features, such as this amiloride-blockable SCC.

Key words: frog skin, current-fluctuation (noise) analysis, prolactin, active Na^+ transport, epithelial Na^+ channel (ENaC), tree frog, *Hyla arborea japonica.*

Introduction

In amphibians, metamorphosis is induced by thyroid hormone *in vivo*. It has long been thought that the juvenile hormone is prolactin, and that its action is to counteract the effect of thyroid hormone (Dodd and Dodd, 1976; Kikuyama et al., 1993). It would therefore be expected that the levels of prolactin would rise in the premetamorphosis and prometamorphosis stages and then drop at the climax of metamorphosis. However, both the serum concentration of prolactin (Yamamoto and Kikuyama, 1982) and the endogenous level of prolactin mRNA (Buckbinder and Brown, 1993; Yamamoto et al., 2000) are low in the premetamorphosis and prometamorphosis stages, and both levels increase at the climax of metamorphosis, suggesting that prolactin has a positive role in the climax stages of metamorphosis, rather than a counteracting role (Kawahara et al., 1999; Huang and Brown, 2000a,b).

Transcellular active Na^+ transport across the skin, measured as the amiloride-blockable short-circuit current (SCC), has been identified in a wide variety of anuran and urodelan genera, including *Rana*, *Leptodactylus*, *Bufo*, *Cynops* and *Ambystome* species (Bentley and Yorio, 1977; Rabito et al., 1978; Bentley and Baldwin, 1980; Hillyard et al., 1982; Takada, 1985; Takada and Komazaki, 1986; Takada and Hara, 1988). This transport develops during the climax stages of metamorphosis in the bullfrog, and is due to the development of an epithelial Na^+ channel (ENaC). Therefore, the appearance of transcellular active Na^+ transport as SCC and/or ENaC is a marker of the development of adult-type features by the skin (Cox and Alvarado, 1979; Hillyard et al., 1982; Takada, 1985).

If prolactin does indeed have a positive role in the progression of metamorphosis, the SCC across the skin should be stimulated by it. In fact, a prolactin-induced increase in the SCC of adult amphibian skin has already been reported (Eddy and Allen, 1979; Takada, 1986).

The activity of the epithelial Na^+ channel (ENaC) located at the apical membrane is the rate-limiting step in transcellular active Na^+ transport in epithelia such as A6 cells, MDCK cells, toad urinary bladder and frog skin. An increase in SCC is considered to reflect an increase in the single-channel current (i) and/or an increase in the channel density (*M*). Blocker-induced current-fluctuation analysis (noise analysis) is a useful method for determining the effect of a hormone on the values of i and *M* when studying ENaC in the epithelium of frog skin, for example (Lindemann and Van Driessche, 1977; Hillyard et al., 1982; Helman et al., 1983; Baxendale-Cox et al., 1997; Els and Helman, 1997). Here, in a study involving current-fluctuation analysis by a two-state model (use of a three-state model proving unsatisfactory with our data), we report that the prolactin-induced increase in the SCC across the skin of the adult tree frog is due to an increase in the open-channel density of ENaC.

Materials and methods

Animals and dissection of ventral skin

Adult tree frogs *Hyla arborea japonica* G were purchased from a local animal supplier in Misato City, Saitama, Japan. The animals were anaesthetized with iced water supplemented with MS-222 (Sankyo Co., Tokyo) and then pithed, before dissecting out portions of ventral body skin.

Electrical measurements

Dissected skin samples were mounted in an Ussing-type chamber fitted with silicone gaskets (5 mm i.d.) to minimize edge damage. Both sides of the skin samples were bathed in aerated Ringer's solution containing (in mmol l^{-1}) 110 NaCl, 2 KCl, 1 $CaCl_2$, 10 glucose and 10 Tris, pH 7.2. The short-circuit current (SCC) was measured continuously under voltage-clamp conditions.

Current-fluctuation analysis was performed before and after application of ovine prolactin (10 μg ml^{-1}; Sigma Chemicals, St Louis, MO, USA). The details of the method used for current-fluctuation (noise) analysis were as previously described (Takada et al., 1999). In brief, the fluctuations in SCC were high-pass filtered (0.05 Hz), amplified (×500) and low-pass filtered (1,024 Hz) to prevent aliasing errors. The signal was sampled at 2,048 Hz. Then, a power-density spectrum (PDS) was calculated for these records using a Digital Spectrum Analyzer (R-9211 A; Advantest, Tokyo). Analysis of the PDS yields the Lorentzian parameters S_o (plateau) and f_c (corner frequency) as follows:

$$S_f = S_o/[(1 + f/f_c)^2] + S_1/f\alpha\,, \quad (1)$$

where S_f is the power of the fluctuations in frequency f, S_1 is the power of the $1/f$ component at 1 Hz and α is the exponent that defines the slope of the $1/f$ component (Hillyard et al., 1982; Helman et al., 1983; Baxendale-Cox et al., 1997; Els and Helman, 1997). The Na^+-channel block by 6-chloro-3,5-diamino-2-pyrazinecarboxamide (CDPC) is assumed to be described by a two-state model of open-block channel kinetics:

$$2\pi f_c = K_{01}[\text{CDPC}] + K_{10}\ , \quad (2)$$

where K_{01} and K_{10} are the blocking and unblocking rate coefficients, respectively, and [CDPC] is the concentration of the blocker, CDPC (Baxendale-Cox et al., 1997; Els and Helman, 1997). The single-channel current (i) and the Na^+-channel density (M: sum of open and blocked channels) were calculated from:

$$i = S_o(2\pi f_c)^2 / 4I_{Na}K_{01}[\text{CDPC}] \quad (3)$$

and

$$M = (I_{Na}2\pi f_c)/(iK_{10})\ , \quad (4)$$

I_{Na} being calculated by subtracting the amiloride-insensitive SCC from the total SCC at each CDPC concentration (Baxendale-Cox et al., 1997; Els and Helman, 1997). The value of i was determined from an extrapolation of the CDPC concentration *versus* i curve to the ordinate (see Fig. 4B). The maximum effect of amiloride on the SCC was achieved at 100 μmol l^{-1}, the remaining SCC being assumed to be amiloride-insensitive. $I_{Na(max)}$ was calculated by subtracting the amiloride-insensitive SCC from the total SCC under CDPC-free conditions.

Statistical analysis

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

Results and Discussion

Prolactin was used at 10 μg ml^{-1} in these experiments since lower concentrations of prolactin induced increases in SCC that were too small for reliable fluctuation analysis (data not shown). Ovine prolactin (oPRL) and *Xenopus* PRL (xPRL) have been reported to have similar effects on larval life-time (Huang and Brown, 2000b) and so oPRL would seem to be useful for analysing the effect of prolactin on adult frog skin. In these experiments, we used CDPC as our channel blocker. This agent is an electroneutral Na^+-channel blocker. Moreover: (1) the y intercept in the corner-frequency plot is larger and more accurate than with amiloride, with which the intercept is near zero, so

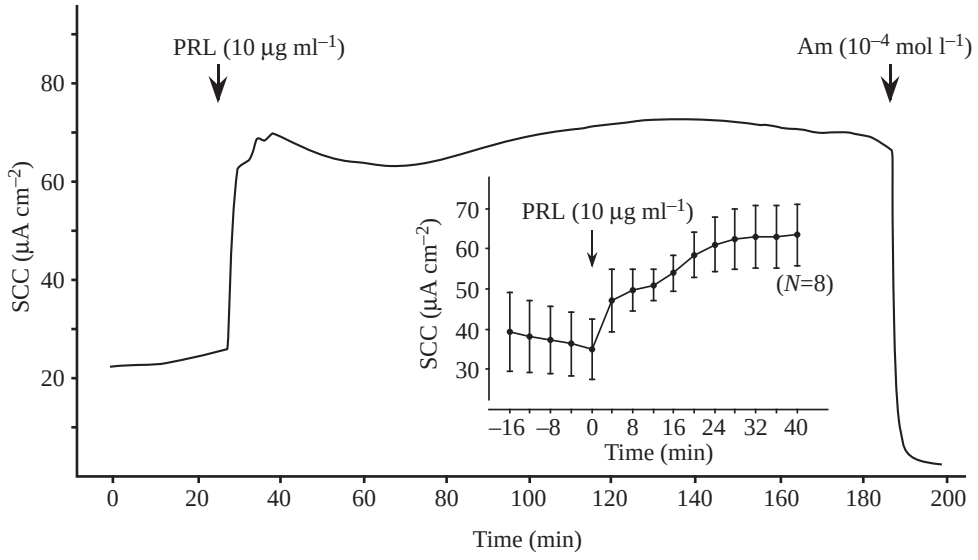

Fig. 1. A typical example of the effect of prolactin (PRL) on short-circuit current (SCC) I tree frog skin (inset shows data for the initial phase of response). Values are means ± S.E.M.; N = number of experiments. PRL (10 μg ml^{-1}) was applied to the basolateral side of the skin and amiloride (Am; 10^{-4} mol l^{-1}) to the apical side, at the times shown.

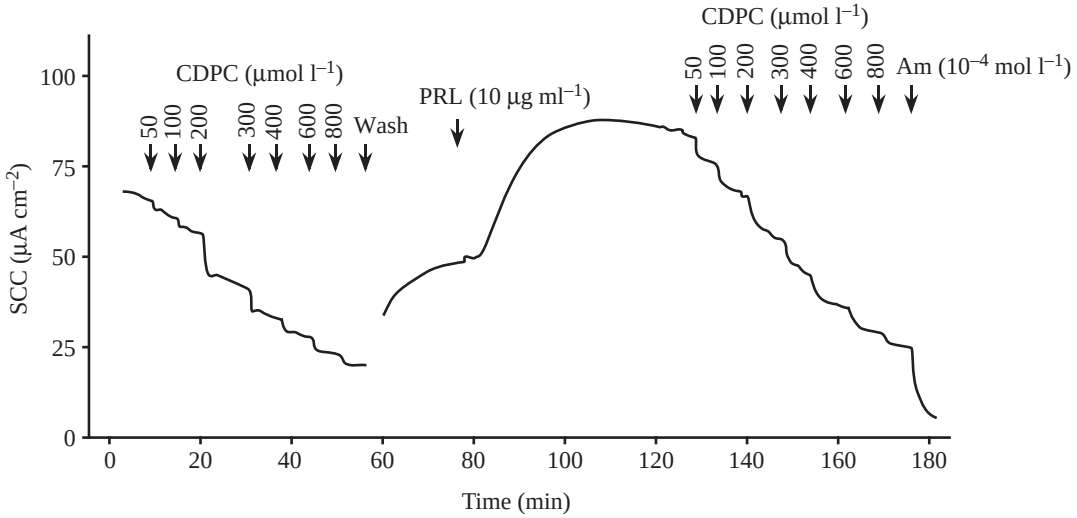

Fig. 2. Typical examples of effect of CDPC in the presence or absence of prolactin (PRL). CDPC (50–800 μmol l^{-1}) was applied to the apical side of tree frog skin at the indicated times and the current-noise power-density spectra were measured at each concentration of CDPC (control condition). CDPC was then washed from the apical side ('wash'). Next, PRL (10 μg ml^{-1}) was applied to the basolateral side of the skin and CDPC (50–800 μmol l^{-1}) was again applied to the apical side. The current-noise power-density spectra were measured at each concentration of CDPC (PRL condition). At the end of the experiment, amiloride (Am; 10^{-4} mol l^{-1}) was applied to the apical side to allow measurement of the amiloride-insensitive SCC.

small differences cause wide variations when determining K_{10} and thus K_m ($=K_{10}/K_{01}$), (2) because of the high K_m, power spectra can be obtained without an almost complete inhibition of SCC, so the I_{Na} values used for calculating *i* are more accurate and also the apical-membrane voltage-gradient becomes less hyperpolarized. This gives a more stable driving force for *i* (Helman and Baxendale, 1990). Therefore, CDPC has advantages for the design of studies involving blocker-induced noise analysis and for the interpretation of data from such studies (Helman and Baxendale, 1990).

Basolateral application of prolactin increased the SCC (Fig. 1); however, apical application had no effect on the SCC (data not shown). This increase in SCC following basolateral application of prolactin was confirmed in SO_4^- and gluconate-Ringer's solutions (data not shown). Prolactin increased the SCC rapidly and the effect was typically maintained for more than 2 h (see example shown in Fig. 1); however, it took 20–30 min for

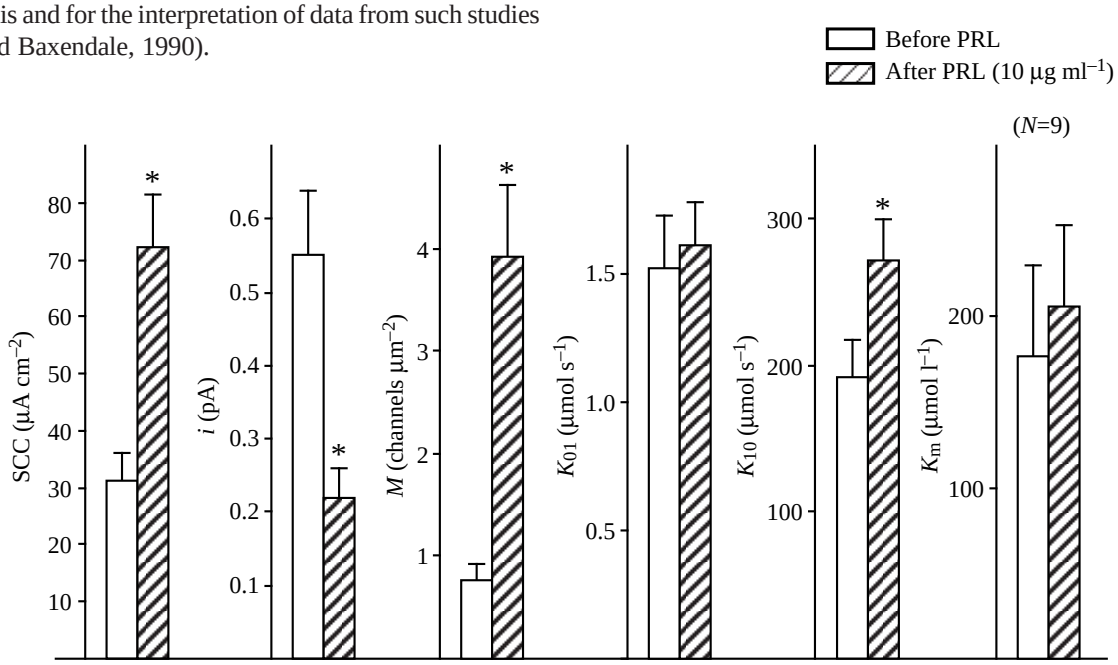

Fig. 3. Summary of effect of prolactin (PRL; 10 μg ml^{-1}) on short-circuit current (SCC; *i*), Na^+ channel density (*M*), K_{01} and K_{10} (blocking and unblocking rate coefficients, respectively) and K_m of adult skin of tree frog. Values are means ± S.E.M.; *N*=9. Asterisks show a significant difference ($P<0.05$) from control values (before PRL).

the response to reach maximum in most cases (Figs 1, 2). In 6/42 cases, the SCC increased rapidly but then desensitized within 10–20 min. We did not use such responses for noise analysis since the SCC needs to be more or less stable for at least 20–30 min for this analysis. As yet, we are not sure why the prolactin-induced increase in SCC desensitized within 10–20 min in these six cases.

Whether the increase in SCC was caused by an increase in the single-channel current (*i*) and/or by an increase in the open-channel density (*M*) of the epithelial Na^+ channel (ENaC) was investigated by current-fluctuation analysis.

Fig. 2 shows a typical example of a staircase experiment in which CDPC was applied before and after an application of prolactin. When the SCC had stabilized after prolactin application, skins were again subjected to a staircase increase in CDPC. Current-noise power-density spectra were measured at each concentration of CDPC, before and after prolactin application.

Prolactin increased SCC 2.6±0.4-fold and *M* 6.1±1.2-fold (Fig. 3), but it decreased *i* to 0.4±0.1 times the control value (Figs 3, 4B). Although K_{10} increased 1.6±0.3-fold, K_m was not significantly different before and after prolactin treatment (Figs 3, 4A).

The decrease in *i* induced by prolactin will presumably have been due at least in part to depolarization of the apical membrane caused by the increase in *M*, since depolarization decreases the single-channel current of ENaC in MDCK cells (Ishikawa et al., 1998). This idea is supported by the effect of steroid hormones and forskolin: both induce an increase in SCC but a decrease in *i* and, in frog skin, the latter is thought to be due to depolarization of the apical membrane (Helman et al., 1983; Baxendale-Cox et al., 1997).

There are two possibilities for the increase in *M*: (1) activation of quiescent ENaC located at the apical membrane or (2) increases in ENaC-trafficking and the cell-surface stability of ENaC. Kemendy et al. (1992) showed that in A6 cells, aldosterone does not increase *M* but does increase *P*o (open channel density). However, other investigators have shown that steroid hormones, antidiuretic hormone (ADH) and forskolin all increase *M* in A6 cells or frog skin, resulting in an increased SCC (Els and Helman, 1997; Helman et al., 1983; Baxendale-Cox et al., 1997; Rosa et al., 2002). Mineralocorticoids increase the activity of serine-threonine kinase in A6 cells and in proximal tubule cells in rats (Loffing et al., 2001; Wang et al., 2001), resulting in an increase in Na^+ transport across the epithelium. Activation of protein kinase C decreases the expression of the β and γ subunits of ENaC in A6 cells (Stockand et al., 2000). These results suggest the possibility that the numbers of ENaC on the apical membrane are regulated by protein phosphorylation. Further research will be needed on whether and how prolactin-signal transduction leads to protein phosphorylation and an increase in *M*.

In conclusion, prolactin has not only a counteracting effect on metamorphosis but also a stimulatory effect on sodium-transport activity (amiloride-blockable SCC) in adult frog skin. The present results suggest an increase in the open-channel density of ENaC, at least following short-term treatment with prolactin.

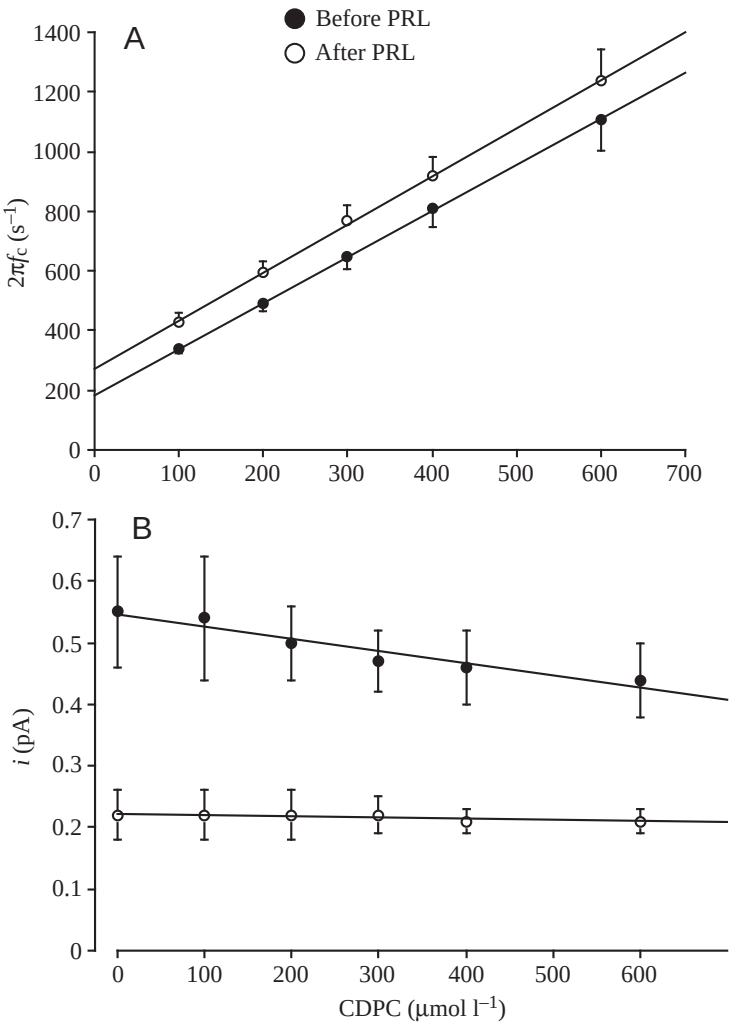

Fig. 4. Blocker-dependent changes in $2\pi f_c$ (see Equation 2) and short-circuit current (*i*). The blocking K_{01} and and unblocking K_{10} coefficients for CDPC are determined from the slope and intercept on the ordinate of corner-frequency plots (A). The single-channel current was calculated by extrapolation of the CDPC concentration *versus i* curve to the ordinate (B). Values are means ± S.E.M.; *N*=9.

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