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A FLUCTUATION ANALYSIS STUDY OF THE DEVELOPMENT OF AMILORIDE-SENSITIVE Na^+ TRANSPORT IN THE SKIN OF LARVAL BULLFROGS *(RANA CATESBEIANA)*

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In the presence of the Na^+-channel blocker amiloride, the short-circuit current across the skins of bullfrog tadpoles in metamorphic stages XIX–XXIV was subjected to fluctuation analysis. The resulting power spectra contained a Lorentzian component of which the plateau value (S_0) decreased while the corner frequency (f_c) increased as the mucosal amiloride concentration was increased from 0.5 to 24 μM. From the linear relationship between the f_c values and the amiloride concentrations it was possible to determine the binding (k'_{01}) and unbinding (k_{10}) constants for amiloride to its receptor on the Na^+ channel. With these parameters as well as short-circuit current and S_0 values, the current through the individual Na^+ channels (i) was calculated (average 0.58 pA). It did not increase significantly during late metamorphosis. The density of Na^+ channels (M) in the apical membrane, on the other hand, increased significantly. It would appear that the increase in short-circuit current which occurs at this time is due primarily to an increase in amiloride-blockable Na^+ channels. Unexpectedly, a Lorentzian component could be fitted to power spectra in amiloride-treated skins (stages XIX–XXI) which showed no amiloride-sensitive short-circuit current. Moreover, the typical increase in f_c with the amiloride concentration did not occur in these animals.

Introduction

The potential difference (p.d.) and short-circuit current (I_{sc}) values measured across the skin of larval bullfrogs are considerably lower in magnitude than those of the adult [1,2]. Cox and Alvarado [2], using techniques to minimize edge damage, found that resistance across the larval skin exceeded 9 $k\Omega \cdot cm^2$ as compared with 1–5.8 $k\Omega \cdot cm^2$ in the adult. In addition, a small I_{sc} was observed which corresponded to the net rate of Na^+ transport across the larval skin. This I_{sc} was not inhibited by amiloride, a pyrazine diuretic which blocks I_{sc} across the adult skin [3,4]. Finally, I_{sc} across the larval skin was greatly stimulated by nystatin, an antibiotic which serves as an ionophore to enhance Na^+ entry into the epithelial cells [5]. It was concluded that the low I_{sc} and high resistance of the larval skin was due to an absence of functional Na^+ channels in the apical membranes and that the appearance of these amiloride-sensitive channels constituted a final step in the development of transepithelial Na^+ transport in this species.

The application of fluctuation analysis to the I_{sc} across adult frog skin by Lindemann and Van Driessche [6] has made it possible to determine rate constants for amiloride binding to, and unbinding from, Na^+ channels. From these parameters it is possible to calculate the density (M) of Na^+ channels (open and blocked) in the apical mem-

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branes, the current passing through a given channel (i) and the probability that the channels will be open for Na^+ conductance (P_o). Thus, the macroscopic I_{sc} can be analyzed as [6,7]:

$$I_{sc} = M \cdot i \cdot P_o \quad (1)$$

P_o is the fraction of channels not occupied by amiloride at a given blocker concentration (see below). It is equal to unity in absence of amiloride.

The present study was designed to document the increase in I_{sc} which occurs during late metamorphosis (stages XXI–XXIV) and to determine, by the use of fluctuation analysis, which of the components i or M are responsible for this increase.

Methods

Bullfrog tadpoles (*Rana catesbeiana*) in hindlimb bud stages I–V [8] were purchased from Carolina Biological Supply Co., Burlington, NC (stock No. L-1493). Upon arrival, the animals were placed in tapwater to which thyroxine had been added to give a concentration of 1 mg/l.

Thyroxine was used to accelerate metamorphosis so that an optimal number of stage XIX-XXIV tadpoles would be available during the time-course of the study. This solution was changed every 2 or 3 days and food (Carolina stock No. 16-4760) was provided with each change. The animals were kept on a 12 h light : dark photoperiod at 21–25°C.

When rudiments of the front limbs began to appear (stages XIX–XX), tadpoles were doubly pithed and the ventral skin was removed along with the attached abdominal musculature as described by Cox and Alvarado [2]. Similar preparations were made at subsequent stages of development such that skins from 17 animals between stages XIX and XXIV were studied.

Skins were mounted between the halves of an Ussing-type chamber specifically designed for fluctuation analysis [9,10]. Soft sealing rings (Sylgard) minimized edge damage and the surface area was small to reduce amplifier noise [11]. The Ringer's solution used in all experiments contained 115 mM NaCl, 2.5 mM $KHCO_3$ and 1 mM $CaCl_2$. The pH was 8.4 at 25°C. After mounting, the chamber was placed in a Faraday cage and the Ringer's solutions were aerated. The p.d. across the skin was clamped to zero with the low-noise voltage clamp apparatus described by Van Driessche and Lindemann [11]. Resistance was measured periodically by clamping the p.d. to +10 mV and dividing this voltage by the difference between the current required to clamp the p.d. to +10 mV and that required to clamp the p.d. to zero. All current and resistance measurements were normalized to 1 cm^2. I_{sc} and resisitance were monitored until stable values for both had been obtained.

At this point fluctuation analysis was conducted as follows. The I_{sc} was initially amplified and then passed through a Rockland 850 filter (48 dB/octave) having a high-pass cut-off at 0.1 Hz and a low-pass cut-off at 850 Hz. The output from this filter was again amplified and the I_{sc} fluctuations were sampled under control of an INTEL 80/30 CPU board while calculations were made concurrently with an INTEL 86/12 CPU board. For analysis, the fluctuations were digitized and three records of 512 points in length were sampled simultaneously on separate channels having different sampling rates. The fundamental frequencies of these channels were 0.2, 0.8 and 3.2 Hz which allowed analysis up to 51.2, 204.8 and 819.2 Hz. In order to avoid aliasing, however, the analog signal of each channel was filtered with a 36 dB/octave low-pass filter having cut-off frequencies of 45, 180 and 750 Hz, respectively. The power spectra from the three channels were calculated with a fast Fourier transform routine written in integer arithmetics. The spectra of 30–40 records from each channel were averaged and plotted together in a double-logarithmic plot. The final averaged spectra were stored on a disc of a PDP 11/34 computer.

The spectra were fitted by the sum, $S(f)$, of a Lorentzian component, $S_0/[1 + (f/f_c)^2]$ and a background noise component, K_b/f^α [10]. In this notation, S_0 (normalized to 1 cm^2) is the plateau value in the lower frequency range and f_c (corner frequency) is the frequency at which the spectral density is one-half of S_0. The spectra shown in Figs. 2 and 3 were plotted with a Hewlett-Packard 7221A plotter.

In the above manner, I_{sc}, resistance and power spectra were recorded under control conditions

(no amiloride) and in the presence of increasing amiloride concentrations.

Results

The I_{sc} across the skins of stage XIX–XX tadpoles averaged 2.64 ± 0.79 $\mu A/cm^2$ (mean ± S.E., $n = 4$) and was not affected by amiloride in the apical Ringer's solution. In five of six tadpoles in stages XXI–XXII the I_{sc} was amiloride-sensitive and clearly dependent on the animals' age. All eight tadpoles in stages XXIII–XXIV showed amiloride-sensitive I_{sc}. These currents are plottted in Fig. 1 as a function of the ratio of the tail length to the total body length. This ratio was chosen because metamorphosis in tadpoles treated with thyroxine is characterized by a continuous reabsorption of the tail [1,12]. Note that an amiloride-insensitive component was consistently found in tadpoles that showed amiloride-sensitive I_{sc}. This current averaged 3.35 ± 0.20 $\mu A/cm^2$ ($n = 12$) and was not significantly different from the amiloride-insensitive I_{sc} of the stage XIX–XX animals. Unlike I_{sc}, no systematic variation was observed in the resistance across the skin as development proceeded. Resistance values ranged from 750 $\Omega \cdot cm^2$ to 4444 $\Omega \cdot cm^2$ with a mean of 2092 ± 229 $\Omega \cdot cm^2$.

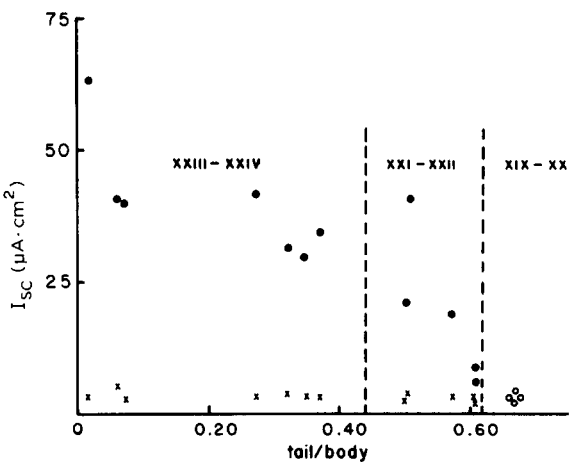

Fig. 1. The increase in I_{sc} as a function of tail reabsorption (expressed as the ratio, tail : body length) during metamorphic stages XIX–XXIV. The open circles refer to skins with no amiloride-sensitive I_{sc} while the closed circles represent those with amiloride-sensitive I_{sc}. The × designates the amiloride-insensitive component of those skins having amiloride-sensitive I_{sc}.

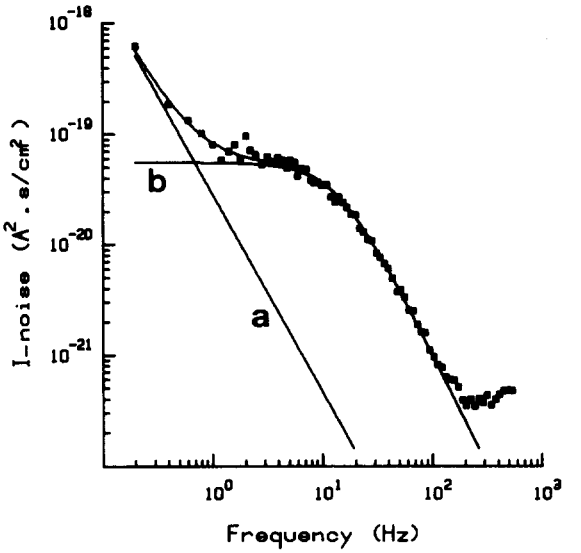

Fig. 2. A typical power spectrum observed with amiloride (6 μM) in the mucosal Ringer's solution along with the linear K_b/f^{α} (a) and Lorentzian (b) components which were fitted to the spectrum by a computer program.

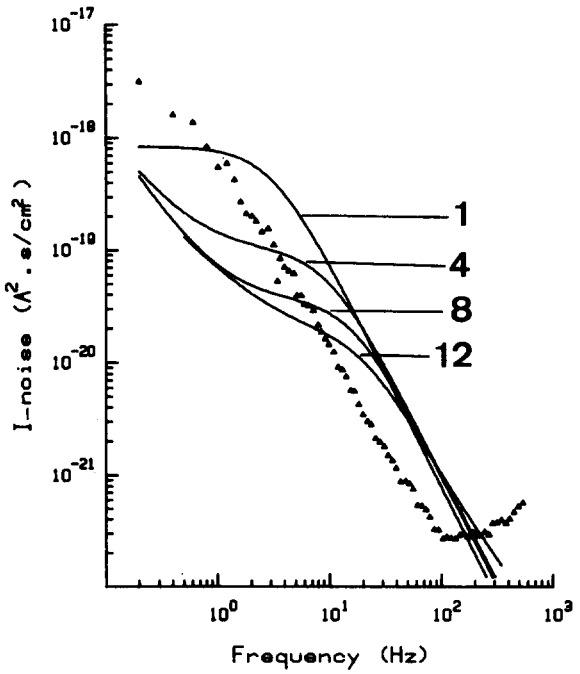

Fig. 3. The power spectra obtained without (▲) and with (lines), increasing amiloride concentrations. For clarity, only the fitted spectra are shown when amiloride was present. Note the decrease in S_0 and the increase in f_c as the amiloride concentration (1, 4, 8, 12 μM – numbers on the curves) was increased.

A typical power spectrum, obtained with 6 μM amiloride in the mucosal NaCl/Ringer's solution, is shown in Fig. 2 along with the K_b/f^α and Lorentzian components which were used to fit the spectrum. A series of power spectra obtained from one preparation under conditions of increasing amiloride concentration is plotted in Fig. 3. Note the absence of a Lorentzian component in the absence of amiloride, while, in the presence of the agent, S_0 decreases and f_c increases as the amiloride concentration is increased. When these changes in S_0 and f_c are compared with the decrease in I_{sc} produced by amiloride, several observations can be made: (1) Both I_{sc} and S_0 decrease in a similar way. This is shown in Fig. 4a and b for tadpoles RC 22 (stage XXIV) and RC 9 (stage XXI). In RC 7 (stage XIX), on the other hand, a Lorentzian component was observed in the presence of amiloride in concentrations above 5 μM even though I_{sc} was not amiloride-sensitive. The S_0 values for spectra from these animals remained fairly stable between amiloride concentrations of 5 and 40 μM. (2) As in the adult frog, f_c increases in a linear manner up to 30 μM amiloride in the most developed (stage XXIV) tadpoles (Fig. 4c). (3) In earlier stages, where amiloride-sensitive I_{sc} is present (RC 9 in Fig. 4c), f_c increases linearly only up to 10–15 μM amiloride and then 'levels off'. (4) Where amiloride-sensitive I_{sc} is not present (RC 7 in Fig. 4c) f_c is already large at low doses of amiloride and does not change markedly with increasing amiloride concentrations reaching as much 40 μM.

The blocking action of amiloride on I_{sc} is described by the reaction:

$$A + R \underset{k_{10}}{\overset{k'_{01}}{\rightleftharpoons}} AR \qquad (2)$$

where A is the amiloride molecule and R is the amiloride receptor at the Na^+ channel. This was recognized to be a pseudo-first-order reaction [6] in which the association proceeds at a rate k'_{01} [A]. The apparent rate constant, k'_{01}, is assumed to be the product of k_{01}, the 'true' rate constant, and the partition coefficient, β, for amiloride at the reaction site. When the complex AR is formed, a reversible closure of the Na^+ channel occurs with the dissociation of AR leading to channel opening.

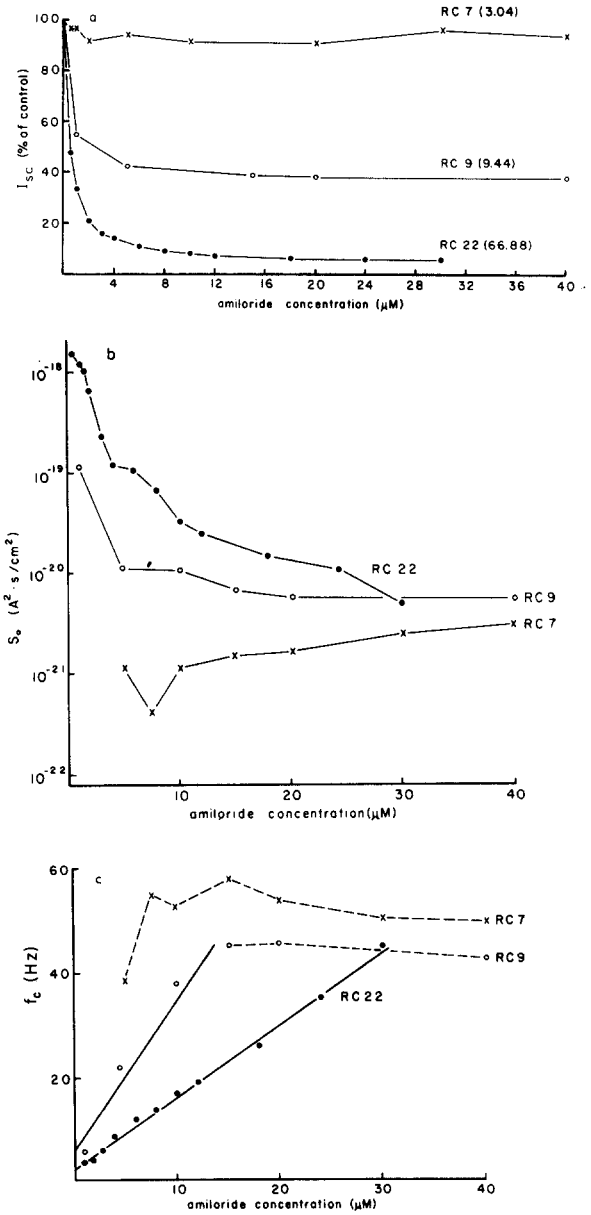

Fig. 4. a. Inhibition of I_{sc} by mucosal amiloride. Note that increasing I_{sc}, in the absence of amiloride (μA/cm^2, value in parentheses), is accompanied by a greater amiloride-sensitive component. Tadpoles RC 22 (●———●), RC 9 (○———○) and RC 7 (×———×) were in developmental stages XXIV, XXI and XIX, respectively. b. The change in the Lorentzian plateau value (S_0) of power spectra produced by increasing the mucosal amiloride concentration. c. The change in corner frequency (f_c) of the Lorentzian component produced by increasing the mucosal amiloride concentration. The solid lines indicate the linear regression used for determining k'_{01} and k_{10}. The dotted lines connect data points which could not be fitted by linear regression.

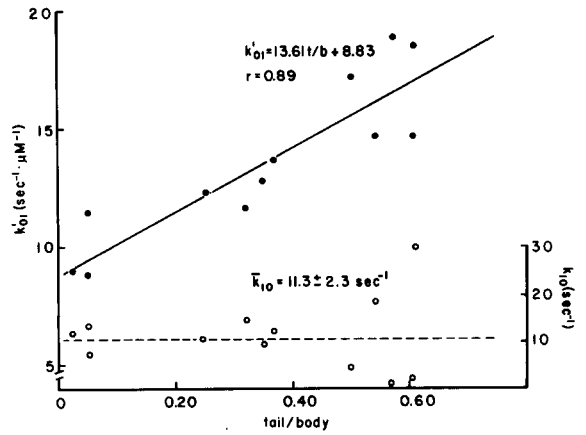

Fig. 5. The effect of development on the binding (k'_{01}) and unbinding (k_{10}) constants of amiloride at its receptor site on the apical surface of the tadpole skin. The solid line is the linear regression of k'_{01} vs. the tail : body ratio, while the dotted line represents mean value for all k_{10} values.

The constant k_{10} describes the rate of AR dissociation. The association and dissociation of amiloride and its receptor thus produce fluctuations in the number of open Na^+ channels, and therefore microscopic current fluctuations around the macroscopic I_{sc}. The rate of current fluctuations is related to $k'_{01}[A]$ and k_{10} in the following equation [6]:

$$2\pi f_c = k'_{01}[A] + k_{10} \tag{3}$$

For skins with amiloride-sensitive I_{sc} a plot of $2\pi f_c$ vs. [A] provides a linear function in which k'_{01} is equal to the slope and k_{10} the intercept. The slope was calculated from the linear regression which best fit the data for each animal (for stages above XXII up to 30 μM amiloride; for stages below XXII up to 15 μM amiloride). The correlation coefficient (r) ranged from 0.98 to 1.00, indicating a very good linear relationship. In Fig. 5 it can be seen that k'_{01} decreases linearly with the resorption of the tail as the animals metamorphose (k'_{01} = 13.61 tail/body + 8.83; $r = 0.89$, $P < 0.01$). On the other hand, k_{10} did not vary with development but rather showed a small random scatter around a mean value of 11.3 s^{-1}.

Once k'_{01} and k_{10} are known, the current passing through each channel (i) can be calculated from the equation:

$$i = \frac{\pi^2 \cdot f_c^2 \cdot S_0}{k'_{01} \cdot [A] \cdot I_{Na^+}} \tag{4}$$

where I_{Na^+} is the I_{sc} at a given amiloride concentration minus the amiloride-insensitive current [6,13]. The i for each animal was calculated as the average value from four amiloride concentrations in the range of 0.5 to 5.0 μM. It was found that i for a given preparation did not systematically change with increasing amiloride concentration.

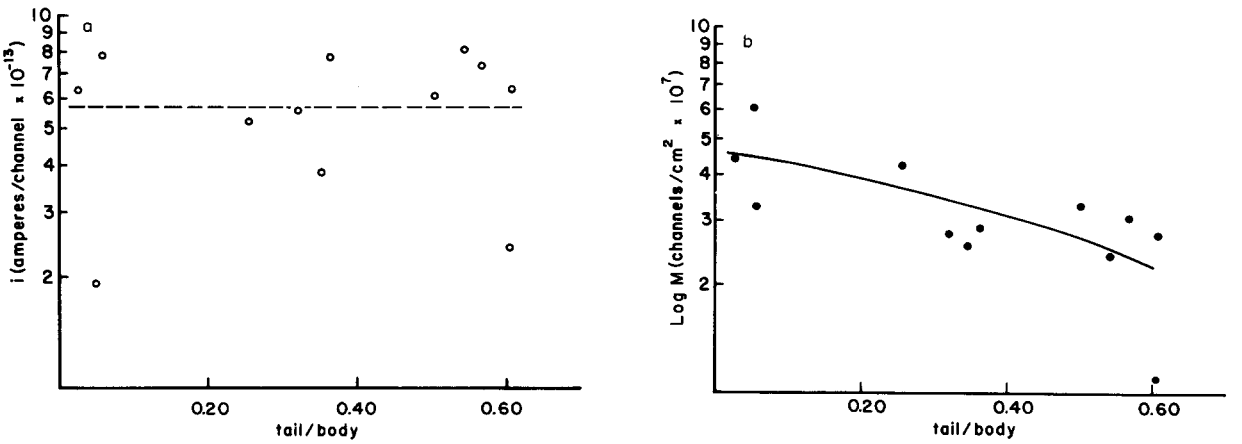

Fig. 6. The effect of development on (a) the single channel current (i) and (b) the density of Na^+ channels (M) in the apical membranes. The dotted line represents the mean value for all i values (= 0.58 ± 0.06 pA/channel). The solid line is the linear regression of M vs. the tail : body ratio, expressed in a semi-logarithmic plot ($M = -4.10\ t/b + 4.68$; $r = 0.73$).

Furthermore, i showed no systematic variation during development (Fig. 6a) despite the increase in amiloride-sensitive I_{sc}. The average value for i was found to be 0.58 ± 0.06 pA ($n = 12$).

Once i had been calculated it was possible to estimate the density of amiloride-blockable Na^+ channels (M) from the equation [6,13]:

$$M = \frac{2\pi}{k_{10}} \cdot \frac{I_{Na^+} \cdot f_c}{i} \quad (5)$$

The average i value was used in calculating M for each tadpole. It can be seen in Fig. 6b that M clearly increases with age. For the range of developmental stages studies in the present paper, a significant correlation between tail reabsorption and M is found ($M = -4.10$ tail/body $+ 4.68$; $r = 0.73$; $P < 0.05$).

Discussion

Taylor and Barker [1] observed that thyroxine, in doses of 10 and 100 mg/ml, produced an accelerated elevation of p.d. across the skins of larval bullfrogs. These animals were quite abnormal morphologically with tail length decreasing before the appearance of limbs. In this same study, it was found that untreated control tadpoles began to display elevated p.d. across the isolated skin at stage XXI. p.d. values in stage XXI–XXII tadpoles were frequently as high as those in stages XXIII–XXV (adult). Assuming that I_{sc} increases in parallel with p.d. (cf. Ref. 2), the data in Fig. 1 for our low-dose thyroxine treated tadpoles are comparable to the untreated controls of Taylor and Barker [1]. In contrast, Cox and Alvarado [2] found I_{sc} in stage XXI–XXIV tadpoles, as a group, to be less than 10 $\mu A/cm^2$ and did not analyze the onset of elevated I_{sc} during late metamorphosis.

Cox and Alvarado [2] did observe much higher resistances than were found in the present study. The surface area of skin in their Ussing chamber was 0.8 cm^2 vs. 0.126 cm^2 in our own and, thus, our preparation is more sensitive to edge damage. The sealing rings in both studies were soft Sylgard; however, Cox and Alvarado [2] used silicone grease between the skin and sealing rings and could use less pressure in securing the skin between the chamber halves. For the calculation of i and M, however, errors due to attenuation of the current noise by resistances in series with the apical membrane [14,15] are unlikely, since the apical membrane resistance by far dominates [2].

The power spectra in stage XXIII–XXIV tadpoles and the calculated values of i and M are very similar to those obtained from adult *Rana esculenta* [6] and from rabbit colon [13]. In stage XXI–XXII tadpoles the binding constant k'_{01} was found to be larger, as seen in Fig. 5, and decreased with development. This observation may be explained by a greater partition coefficient for amiloride in the apical membranes of the epithelial cells of stage XI–XXII tadpoles. It is also possible that the membrane environment of the Na^+ channel changes with metamorphosis in such a way that the intrinsic rate constant, k_{01}, would depend on the age of the tadpole.

In contrast to the relatively constant values for i, M is seen to increase significantly with development, as does the amiloride-sensitive I_{sc}. The variability in values for M, calculated from Eqn. 5 arises largely from the imprecision associated with the determination of k_{10} (since the plot of $2\pi f$ versus amiloride concentration intersects the ordinate very close to zero) and of I_{Na^+} at amiloride concentrations well above K_m (the Michaelis constant for amiloride inhibition of I_{sc}); see also Ref. 13. We therefore have to limit ourselves to a semi-quantitative view when comparing the age-dependence of M and I_{Na}.

Within the limits of fluctuation analysis we have demonstrated that the increase in amiloride-sensitive I_{sc} in developing tadpoles correlates with an increase in the number of amiloride-blockable channels, as suggested earlier by Cox and Alvarado [2]. The presence of a Lorentzian component in the power spectra in skin of stage XIX–XX tadpoles treated with amiloride (Fig. 4b, c) shows that an amiloride-binding site is present prior to the appearance of amiloride-sensitive I_{sc}. In a related study, Hillyard et al. [16,17] have shown that amiloride and benzimidazolylguanidinium, an amiloride analogue known to stimulate I_{sc} in the adult skin [18], both induce a Lorentzian component in the power spectrum of stage X–XIX tadpoles and produce small but only transient increases in I_{sc}. It was hypothesized that an embryonic Na^+ channel exists which may give rise to

the amiloride-blockable channel later in development. In addition, spontaneous Lorentzian components could be observed in power spectra obtained with K^+ as the primary cation in the mucosal Ringer's solution with skins from stage X–XIX tadpoles. This noise was seen in absence of amiloride or benzimidazolylguanidinium but could be much enhanced by these agents, while the respective corner frequencies decreased. This is opposite to the behaviour expected from blocker noise and appears at very large amiloride concentrations (see Fig. 4c) only in the youngest tadpoles. The Lorentzian components for both Na^+ and K^+ could be abolished by adding the well-known K^+-channel blockers, Ba^{2+} or tetraethylammonium to the mucosal Ringer's. Since Ba^{2+} is also known to block the apical K^+ channel in adult *Rana temporaria* skin [10] it was speculated that the non-selective embryonic channel may be a common precursor for both Na^+ and K^+ channels in the apical membranes. Fluctuation analysis has therefore been a useful tool for quantifying the presence of amiloride-blockable Na^+ channels and may be of great help in investigating any differentiation which may occur in the embryonic development of epithelial Na^+ and K^+ channels.

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