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Changes in Ionic Conductances and in Sensitivity to Amiloride during the Natural Moulting Cycle of Toad Skin (*Bufo viridis*, L.)

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Summary. The resistance of the apical membranes of toad skin (*Bufo viridis*) was measured during its natural moulting cycle using a fast flow technique. The skin behaved in all periods of the moulting cycle as a nearly perfect sodium electrode. In the presence of amiloride (10^{-4} M), the total resistance of the same skin was identical with solutions which contained either sodium or potassium. The resistance of the skin with potassium was sensitive to amiloride in the period just after moulting. The resistance of skins which were made shunted by treating them with urea on the outside was insensitive to amiloride in solutions containing potassium; a small effect was still observed with sodium. It is suggested that the transient sensitivity to amiloride, with potassium, is the result of differentiation of the sodium specific sites at the apical membranes of the skin.

Shedding of the *stratum corneum* of the skin in toads and frogs is accompanied with considerable changes in physiological characteristics of the skin (Jørgensen, 1949; Nielsen, 1969; Hviid-Larsen, 1970; and others). The increase in short-circuit current following slough formation was accounted for by an increase in the rate of active sodium transport and an increase in the passive permeability to sodium (Hviid-Larsen, 1970).

The fast flow technique, which has been developed by Lindemann and co-workers (1972), allows a direct study of the apical functional membranes of the skin by recording electrical changes while rapidly changing solutions over it. This technique has been applied for a study of the characteristics of passive permeability of the outer barrier of toad skin during its natural moulting cycle.

Materials and Methods

Toads, *Bufo viridis*, were collected in Israel. Animals of both sexes were kept separately at 21 ± 2 °C in deionized water (2–3 cm deep), and were not fed during the experimental

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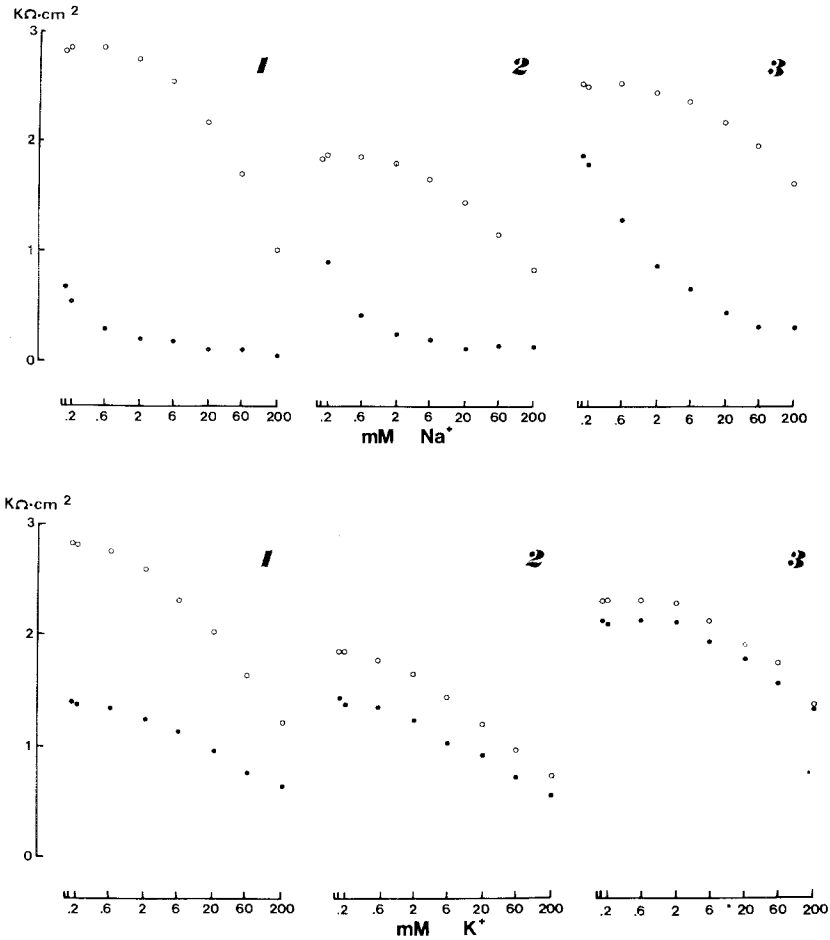

Fig. 1. Instantaneous *R-c* curves (resistance as a function of concentration) of toad skin epithelium, during the natural moulting cycle. Period *1*: time when slough just came off; period *2*: 3–5 hours later; period *3*: time in between two moults. (*A*): the resistance measured in solutions with increasing concentrations of sodium; (*B*): the resistance measured in solutions with increasing concentrations of potassium. Open circles represent determinations which were taken in the presence of 10^{-4} M amiloride. Skins were preequilibrated with 1 mM Ca-gluconate, 5 mM Tris-sulfate, pH = 6.0, on the outside; and sulfate-agar Ringer's on the inside

period. Shedding was followed by inspecting red lipstick marks on the neck of the animals, which disappeared upon shedding. The toads moulted about every 4–5 days under these conditions at regular intervals. Animals were picked up at a desired timing along their individual moulting cycles after 7–8 successive observed moults. They were double pithed, and the abdominal skin was dissected and mounted in the 'fast flow' apparatus, as described by Lindemann *et al.* (1972). In brief, an area of 1 cm^2 of the skin formed in the apparatus the bottom of a shallow flow channel (1 mm high). Solutions which flow through the

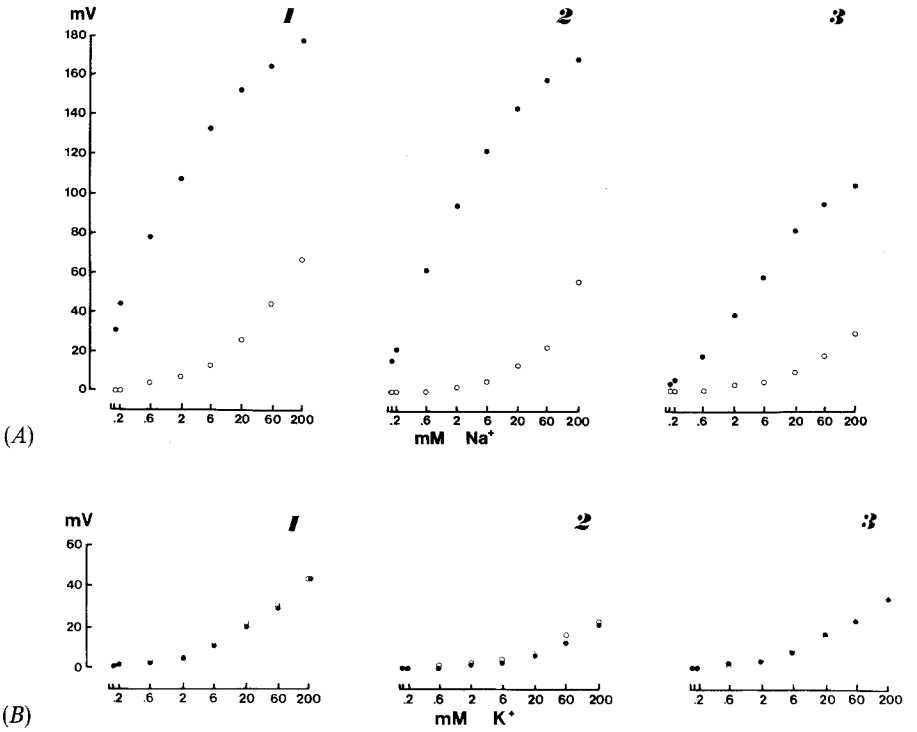

Fig. 2. Voltage responses of toad skin epithelium to changes in concentrations of sodium (*A*), or potassium (*B*) in 3 periods along the natural moulting cycle. Same skins, timing, and solutions as in Fig. 1. Open circles are responses which were recorded in the presence of 10^{-4} M amiloride

channel can be changed from one to another quickly by means of an electro-magnetic switch. The outer surface of the skin was equilibrated for an hour with a solution containing 1 mM Ca-gluconate and 5 mM Tris-sulfate at pH = 6.0.

The instantaneous concentration-dependence of skin resistance (*R-c* function) was determined during short exposures of its surface to solutions containing either sodium or potassium, in concentrations up to 200 mEquiv/liter, and gluconate as the only co-anion. Resistance was determined by applying a square pulse current of 0.5 sec duration, after a steady-state potential was attained following fast changes of solution over the skin.

The amiloride sensitivity of the shunt pathway (Ussing & Windhager, 1964), was studied in a conventional "Ussing" type chamber. After 1 hr equilibration with sulfate-Ringer's [(in mEquiv/liter) Na_2SO_4 55.5; K_2SO_4 0.95; $NaHCO_3$ 2.38; $CaSO_4$ 0.89; pH = 7.0], on both sides of the skin, steady-state resistance was determined by passing a depolarizing current (100 μA/3 cm^2) across the skin, and measuring the consequent drop in potential. Only in these experiments osmotic concentration was maintained by substitution with $MgSO_4$ when concentrations lower than 115 mEquiv/liter of sodium or potassium were used.

All experiments were carried out at room temperature. Chemicals were of analytical grade. Amiloride was from Merck, Sharp & Dohm, N.J.

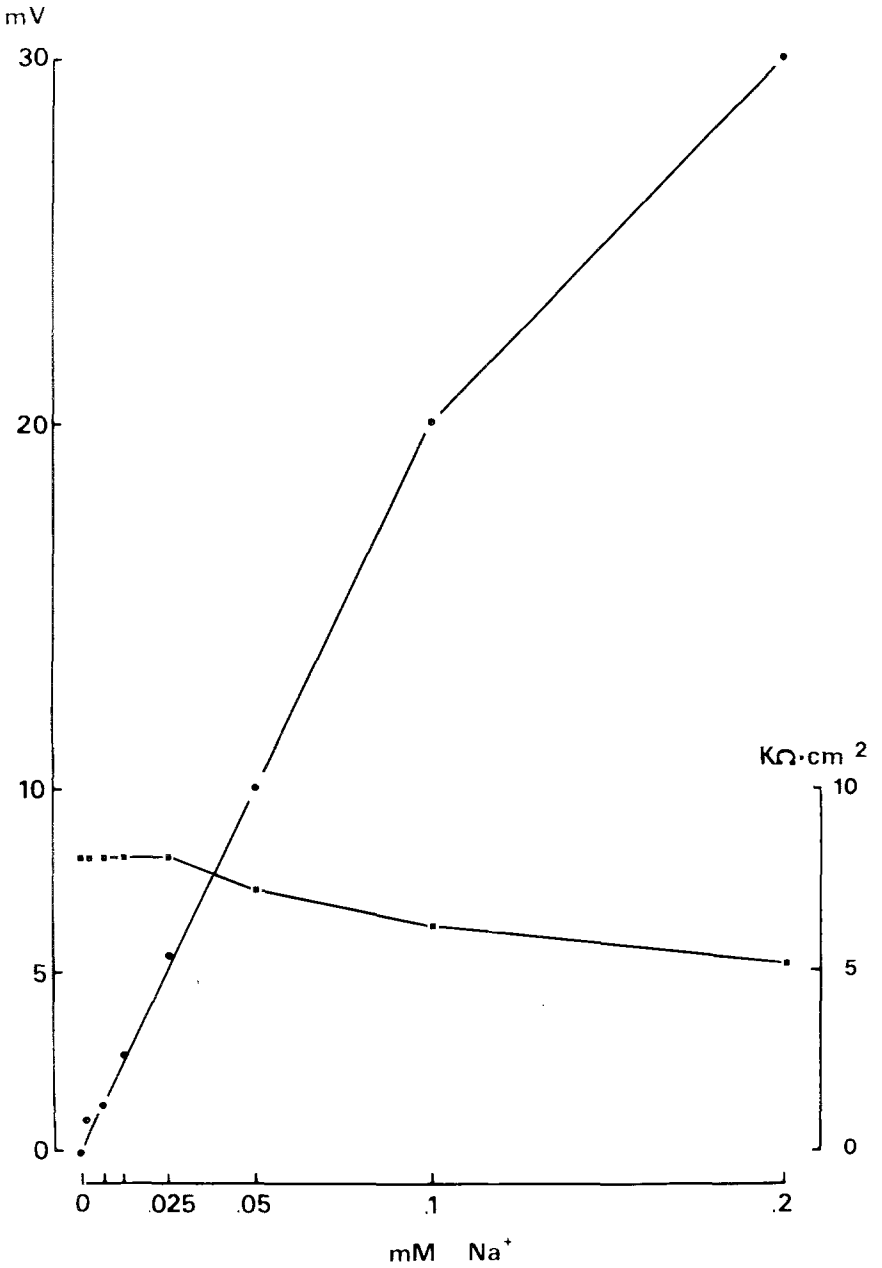

Fig. 3. Voltage response and changes in resistance of toad skin epithelium, just after shedding, to changes in sodium concentrations. Skin was preequilibrated with 1 mM Ca-gluconate, 5 mM Tris-sulfate, pH = 6.0, on the outside, and sulfate-agar Ringer's on the inside. Resting potential was 60 mV, positive outside; voltage response, which was negative, is plotted in absolute values (filled circles). Filled squares show the actual resistance at each concentration of sodium

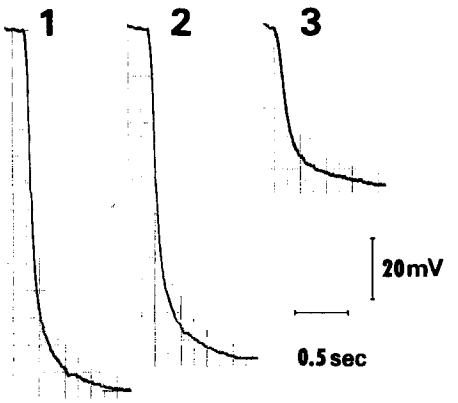

Fig. 4. Voltage response of toad skin epithelium to sudden introduction of 6.0 mM Na-gluconate. Original records from experiments on the same skins of Figs. 1 and 2. Times to complete half response ($t_{1/2}$) are (*1*): 50 msec; (*2*): 75 msec; (*3*): 125 msec. Resting potentials were positive outside. (*1*): +55 mV; (*2*): +55 mV; (*3*): +30 mV. Potential became more negative in response to the introduction of solution with sodium, which is plotted downwards

Results

Fig. 1 shows a plot of the resistance of three representative skins (out of 10 skins which had similar characteristics) as a function of concentrations (*R-c* function) of either sodium (*A*) or potassium (*B*). Measurements were taken also in the presence of 10^{-4} M amiloride. The three parts in the two diagrams (*A* and *B*) are measurements which were taken on skins from three animals at time when slough just came off (period *1*) , 3–4 hr after shedding (period *2*)], and in the middle of the moulting cycle, between two moults (period *3*).

The principal feature of these figures is the sensitivity of the resistance of the skin in the presence of potassium to amiloride (periods *1* and *2*, Fig. *1B*). This sensitivity disappeared shortly after moulting, and was accompanied by a slight increase in the total resistance of the skin (periods *2* and *3* in Fig. 1*A* and *B*).

Voltage responses of the same skins, in solutions containing either sodium or potassium, are shown in Fig. 2*A* and *B*. The skin always behaved as a nearly perfect sodium electrode, over the range of concentrations used. The response to potassium was rather small. Amiloride did not have any effect on the voltage response in solutions containing potassium.

Moulted skins were highly sensitive to very small concentrations of sodium. This can be seen in Fig. 3: 0.91 mV voltage deflection was

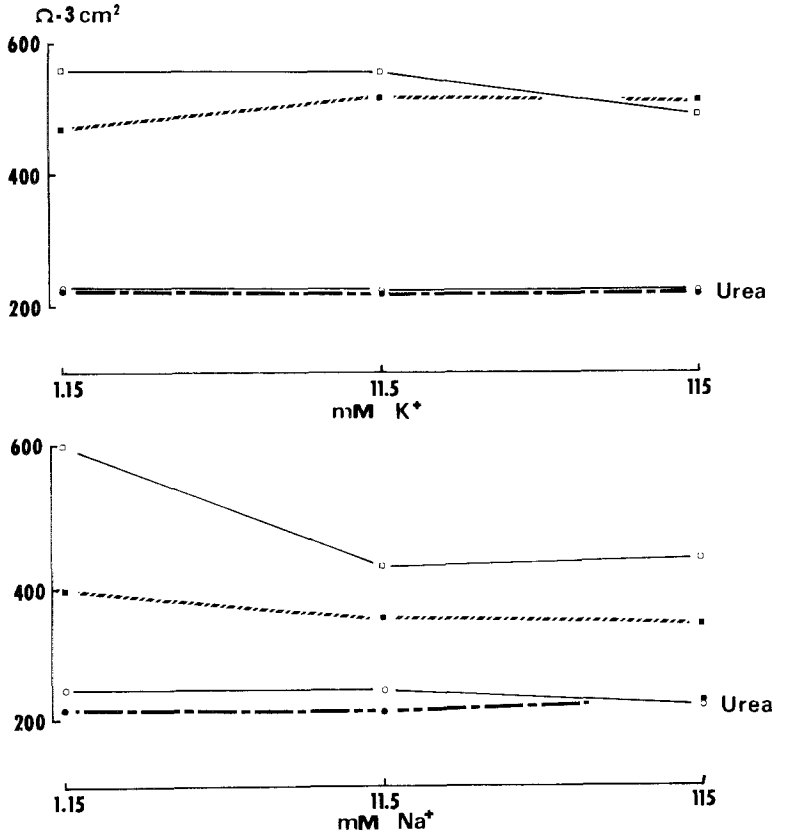

Fig. 5. Effect of urea treatment on sensitivity to amiloride in toad skin epithelium. Skins were preequilibrated in sulfate-Ringer's (pH = 7.4) on both sides. Resistance was then determined at 3 concentrations of either sodium or potassium on the outside (broken lines), and again in the presence of 10^{-4} M amiloride (continuous lines). The same determinations were repeated after the addition of 230 mM urea to the outside of the skin

detected in response to 1.6×10^{-6} M of sodium (0.0016 mEquiv/liter) in the solution bathing a newly appearing skin, just after shedding; a concentration not less than 0.05 mEquiv/liter of sodium was needed to detect a change in conductance of the same skin.

The speed of the voltage response of the same three skins from Figs. 1 and 2, is shown in Fig. 4. Both the magnitude of the absolute voltage response and its speed were decreased considerably in the intermoult skin. $t_{\frac{1}{2}}$ increased from 50 msec just after moulting (period *1*) to 125 msec in the intermoult skin (period *3*).

The amiloride sensitive potassium resistance could arise from either the shunt pathway (rearrangement of junctions) or differentiation of

the sodium sites at the outward facing membranes as they move outwards. These alternatives were investigated using the method of Ussing and Windhager (1964). Results from one of the skins thus studied, are shown in Fig. 5. It can be seen that urea treatment, which lowered the resistance of the skin to about half of its original value ("shunted" skins), made the skins insensitive to amiloride when the solution bathing the outer surface was of potassium; a small effect was observed, however, with solution containing sodium. The shunt pathway predominates in "shunted" skins under urea treatment; amiloride did not have any effect under these conditions since there are no sites for potassium there.

Discussion

The results presented in this paper provide direct evidence that the changes in the permeability of toad skin during moulting take place mostly at the apical membranes of the cells.

The timing of period *1* in Figs. 1 and 2 in this study may be correlated with the "activation period" of Nielsen (1969) in the frog, and the "final stimulatory period" of Hviid-Larsen (1972) in the toad, in their aldosterone-stimulated skins. Three to five hours later, the permeability characteristics of the skin become very similar to intermoult skins.

In the double barrier model, Koefoed-Johnsen and Ussing (1958) visualized the skin to be functionaly asymmetric in its selectivity towards sodium and potassium, on either of its sides. The "outward facing membrane" is believed to be located just beneath the cornified layer, whereas the "inward facing membrane" should correspond to the membranes limiting the extracellular spaces throughout the epithelium (Ussing & Windhager, 1964; Farquhar & Palade, 1966). The functional asymmetry should therefore be assumed to be developed during each moult, as cells move outwards; it should not be an inherent property of the cells of the *stratum germinativum,* or of any other layer of the epithelium (Hviid-Larsen, 1972; Nielsen, 1973).

The major finding of this study is the great sensitivity of the resistance of the skin to amiloride, in solutions containing potassium, during shedding of the *stratum corneum;* this sensitivity disappeared shortly after moulting (periods *2* and *3* in Fig. 1*B*). The total resistance of the skin in all periods of the moulting cycle in the presence of amiloride was, however, more or less the same (*compare A* and *B* in Fig. 1). This is to be expected if the sites reacting with amiloride were the same ones.

During the moulting phase, these sites underwent differentiation to become specific to sodium, while some of them still reacted with potassium.

Contamination of solutions with sodium does not seem to be possible in these experiments. Skins have been found to be highly sensitive to very small concentrations of sodium, with potential responding to even lower concentrations than conductance (Fig. 3). Any contamination of sodium in the experiments where potassium was used, should have then been detected, if it was there.

The amiloride-sensitive resistance could possibly be the result of junctional rearrangement during the "activation period" of the moulting cycle. The experiment with "shunted" skins (Fig. 5) does not support this possibility, since under these conditions, where the shunt pathway was more accessible to the outside (Ussing, 1965), amiloride was without effect on the resistance with potassium while still somewhat effective with sodium. It seems, then, that sites on the apical membranes of the epithelium play the major role in permeability changes during moulting in the toad. A similar conclusion was drawn by Nielsen in the frog (1973).

The delay in voltage response of the skin in the intermoult period (Fig. 4) is in accord with findings of others (Lindemann *et al.*, 1972; Nielsen, 1973). The gradual increase in the delay of voltage response after moulting could result from thickening of the unstirred layer, which is composed of extracellular material covering the skin.

The newly appearing skin, which is highly conductive (Fig. 3), is also extremely sensitive to amiloride (Fig. 1*A* and *B*; Katz, 1973). Nielsen and Tomilson (1970) reported a reduced sensitivity to amiloride in frog skin one to three hours following removal of the *stratum corneum*. This descrepancy might have arisen from the difference in the methods used, if it is not due to a species difference. The latter authors removed the *stratum corneum* mechanically after moulting was induced with aldosterone; there might not have been, then, enough time for the complete differentiation of the sodium specific sites.

The time course of the changes in permeability, which were verified in this study, correlate fairly well with other physiological parameters which were measured by others (Jørgensen, 1949; Hviid-Larsen, 1972). Structural observations showed, however, marked changes in cell junctions (zonulae occludents) during the shedding phase (Budtz & Larsen, 1975); from the experiments on shunted skins in this study (Fig. 5), it does not seem that the tight junctions (zonulae occludents) are responsible for the observed changes in specific permeabilities to sodium and potassium.

It would be interesting to know the mechanism and trigger for the differentiation of these sites.

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