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Sodium Flux in the Apical Membrane of the Toad Skin: Aspects of Its Regulation and the Importance of the Ionic Strength of the Outer Solution upon the Reversibility of Amiloride Inhibition

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Summary. Injection of small pulses of concentrate solutions of salts or drugs into the outer bathing fluid led to sudden increases of its solute concentration. Vigorous stirring of the outer bathing solution was used to minimize the thickness of the unstirred layer adjacent to the outer skin surface. Pulses of 1 M NaCl injected into the outer compartment induced sharp increases of the SCC following a time course variable with the magnitude of the pulse and the particular condition of each skin. Comparison of the spontaneous decline of the SCC with the decline induced by a small dose of amiloride, where an increase in R was observed, indicates that the spontaneous decline cannot be explained simply as a reduction of the Na permeability of the apical membrane by self-inhibition of feedback inhibition of the apical membrane Na channels. Reduction of the driving force for Na movement into the epithelial cells must play an important role in the process. Reversibility of the amiloride inhibition of the SCC was highly dependent upon the ionic strength of the solution used to rinse and wash out the inhibitor from the outer skin surface. With H_2O, the amiloride molecules washed out slowly as compared to NaCl or KCl solutions. Na or K have the same ability to dislodge the amiloride molecules from their binding sites. This effect is apparently of a purely electrostatic nature.

Key Words toad skin · apical membrane · transport regulation · amiloride · Na transport

Introduction

Na transport across amphibian skins is a transcellular two-step process (Koefoed-Johnsen & Ussing, 1958). The Na-entry step takes place across the Na channels of the apical membrane, and the exit is accomplished by the Na pump, the Na, K-ATPase, located at the basolateral membrane of the epithelial cells.

The Na permeability of the apical membrane, $(PNa)_o$, of amphibian epithelia (skin and urinary bladder) and of other epithelial membranes is under the control of several mechanisms (Ussing, 1978). Hormonal control is clearly exerted by various physiological messengers (Crabbé, 1964; Edelman & Fimognari, 1968; Fuhrman & Ussing, 1951; Leaf, 1965). Among them, the antidiuretic hormone and aldosterone are the best-studied examples. These two hormones seem to increase $(PNa)_o$ by increasing the density of apical membrane Na channels (Li et al., 1982; Palmer et al., 1982). For each hormone the effect is mediated by a different chain of events. The hormonal actions exemplify the control of $(PNa)_O$ exerted by large feedback loops involving the animal as a whole.

A short or local feedback loop, the feedback inhibition of the apical membrane Na channels was also described (Erlij & Smith, 1973; Leblanc & Morel, 1975) and the cytosolic Na or Ca concentrations have been proposed to be the regulatory signal (Erlij & Smith, 1973; Leblanc & Morel, 1975; Grinstein & Erlij, 1978; Chase & Al-Awqati, 1981; Bevevino & Lacaz-Vieira, 1982). This regulatory mechanism provides a means for the transepithelial Na transport to limit itself.

An open loop regulation, the Na self-inhibition of the apical membrane Na channels (Cereijido et al., 1964; Lindemann & Gebhardt, 1973; Fuchs et al., 1977) also seems to contribute to an adjustment of the rate of Na entry across the apical membrane not associated to the rate of Na extrusion by the Na pump.

Several drugs have been tested and shown to interfere with $(PNa)_o$, increasing (Zeiske & Lindemann, 1974; Li & DeSousa, 1979) or decreasing (Nagel & Dorge, 1970, Salako & Smith, 1970; Cuthbert & Wong, 1972) it, or altering the intrinsic patterns of the regulatory mechanisms (Zeiske & Lindemann, 1974).

The present study, in the line of a previous work (Procopio & Lacaz-Vieira, 1977), aims at further insight into the processes and mechanisms involved in the translocation of Na ions across the apical boundary of the skin in conditions of low ionic strength of the outer bathing solution.

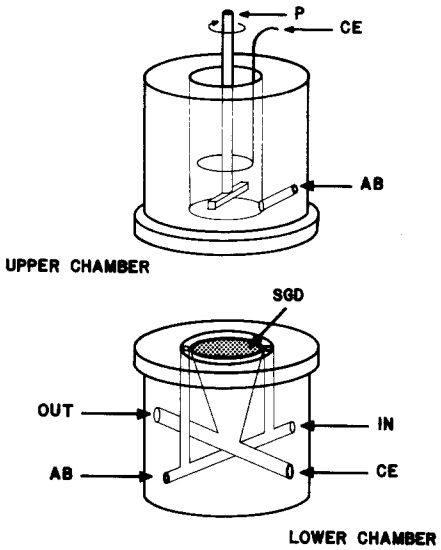

Fig. 1. Diagram of the chamber. *CE* = Ag-AgCl current electrodes; *AB* = KCl agar bridges; *IN* = solution inlet; *OUT* = solution outlet; *SGD* = sintered glass disc; *P* = propeller

Materials and Methods

Abdominal skins of the toad *Bufo marinus ictericus* were used. The animals were double pithed prior to the skin removal.

Pulse Chamber

A diagram of the chamber is shown in Fig. 1. A piece of skin of 3.14 cm^2, with the epithelial side facing upwards, was mounted horizontally. Special precautions were taken to prevent the effect of skin edge damage by using hemichambers provided with a silicone grease gasket (high vacuum grease) located at the internal rim of the hemichamber surface in contact with the epithelial side of the skin. The outer bathing solution was vigorously stirred at 3000 rpm by a stainless steel propeller with the paddles placed 1.5 mm above the outer skin surface. This vigorous stirring had two important functions: a) To minimize the thickness of the unstirred layer adjacent to the outer skin surface. The presence of this unstirred layer drastically affected the values of the skin electrical potential difference when dilute salt solutions bathed the outer skin surface (Carmona & Lacaz-Vieira, 1979). *b*) To rapidly equilibrate the concentration of the outer bathing medium when pulses of concentrate solutions were injected into this medium. The efficiency of mixing was evaluated electrometrically by means of an Ag-AgCl electrode built with its sensing spot leveled with the surface of a thin Plexiglas® disc mounted in place of the skin. 18 msec was the observed half-time for the concentration to reach equilibrium at the electrode surface when a pulse of 1 M NaCl was injected into the outer compartment, previously filled with 5 ml of 1 mM NaCl.

Voltage Clamp

A conventional voltage clamp with continuous feedback was used. Saturated KCl agar bridges and saturated calomel half-cells were used to monitor the electrical potential difference across the skin. The tip of the outer bridge (50 μm in diameter) touched the outer skin surface tangentially, so that a negligible layer of solution was interposed between the sensing bridge and the outer skin surface. A similar precaution was taken regarding the inner sensing bridge. Ag-AgCl electrodes, adequately placed to give a uniform current density across the skin were used for current passing. The clamping current was continuously recorded in a strip chart recorder.

Experimental Procedure

The skin was bathed on the inner side by NaCl-Ringer's solution renewed approximately every 5 min by a continuous flow across the inner compartment. At the beginning of each experimental run the outer compartment was rinsed several times with distilled water and finally filled with 5 ml of distilled water. The voltage-clamp was then set to the clamp mode. After a short interval of time, a pulse of 1 to 100 μl of 1 M NaCl solution was injected into the outer bathing solution suddenly increasing its salt concentration. Pulses of 1 to 100 μl of amiloride 10^{-4} M, were also injected into the outer bathing fluid at proper moments. The transient changes of SCC associated with the pulses were recorded and analyzed. The NaCl-Ringer's solution had the following composition, in mM: NaCl 115, $KHCO_3$ 2.5 and $CaCl_2$ 1.0, with an osmolality of 220 mOsm/kg of water and a pH of 8.0 to 8.2 after aeration. The results are presented as mean ± standard error of the mean.

Abbreviations and Conventions

$(X)_o$—indicates concentration of species X in the outer bathing medium.
SCC—short-circuit current. The convention is such that a positive SCC corresponds to the transport of positive charges across the skin from the outer to the inner bathing solution.
PD—skin electrical potential difference.
R—skin electrical resistance. Calculated from the deflections of the clamping current induced by pulses of ± 20 mV, as $R = \Delta V/\Delta I$, where ΔV and ΔI are the corresponding changes in the skin electrical potential difference and the clamping current, respectively.

Results

A. Short-Circuit Current with Distilled Water Bathing the Outer Skin Surface

Skins bathed by H_2O on the outer surface and NaCl-Ringer's solution on the opposite side, consistently show a null SCC which is a stable condition that could be followed for long periods of time. In a few cases, a negative SCC of small magnitude (normally less than 2 $\mu A\ cm^{-2}$), stable or displaying a slight tendency to increase to more negative values could be seen. Positive SCC's were never observed. The stability of the null SCC suggests that no appreciable salt leak takes place across the apical boundary of the skin.

In rare occasions, very leaky skins were found and indication of salt accumulation in the outer compartment was detected. In these skins a negative SCC that increased with time and could be abolished by a continuous flow of H_2O across the outer compartment was observed. So far, no attempts were made to identify the nature of the ions accumulating in the outer compartment. These leaky skins had no visible indication of macroscopic lesions on the epithelial surface when examined optically under high magnification, or edge damage. All were discarded for further studies.

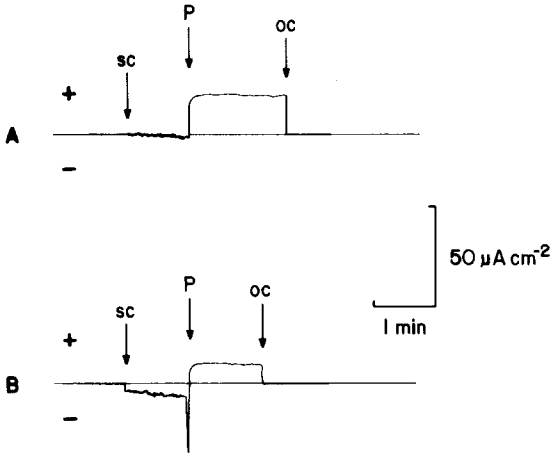

Fig. 2. SCC time course following the injection of a pulse of 2 μl of 1 M NaCl solution into the outer compartment, leading to a sudden increase of $(NaCl)_o$ from zero (H_2O) to 0.4 mM. Initially, the skins were bathed by H_2O on the outer surface. SC: the voltage-clamp was set to the clamp mode. P: a pulse of NaCl was injected into the outer bathing medium, kept vigorously stirred by a rotating propeller. OC: the voltage-clamp was turned off. The + and − signs indicate the direction of positive and negative SCC, respectively. In *A*, a skin with a null SCC when bathed by H_2O on the outer surface is presented. The pulse of NaCl induced a fast positive increase of SCC. In *B* a different skin shows a negative SCC when bathed by H_2O on the outer surface. The pulse of NaCl led to a fast and sharp negative peak followed by a positive deflection to a stable value

B. Time Course of the Short-Circuit Current Following the Injection of Pulses of NaCl into the Outer Compartment

Pulses of 1 to 100 μl of 1 M NaCl solution injected into the outer compartment caused sudden increases of $(NaCl)_o$, as described in Materials and Methods. The time course followed by the SCC was a function of the pulse magnitude and the particular condition of each skin.

1. *Small Pulses*

Skins displaying a null SCC with H_2O bathing their outer surfaces normally respond to a small NaCl pulse with a positive deflection of SCC, which reaches a plateau with a half-time of a few seconds (Fig. 2*A*). For our purposes, a small pulse is one which increases $(NaCl)_o$ from 0 up to 5 mM. The few skins showing a small negative SCC when bathed by H_2O on the outer surface, normally present a biphasic response to a small NaCl pulse, characterized by a sharp negative peak of short duration preceding the positive deflection (Fig. 2*B*).

2. *Series of Pulses*

A series of small NaCl pulses, or of pulses of increasing magnitude, when injected into the outer compartment cause the SCC to increase in a staircase fashion, as shown in Fig. 3*A*. A plot of SCC as a function of $(NaCl)_o$ shows a nonlinear dependence, as depicted in Fig. 3*B*, for the same data shown in Fig. 3*A*.

3. *Large Pulses*

Large pulses of NaCl when injected into the outer compartment, increasing $(NaCl)_o$ from zero to values normally above 15 mM, cause the SCC to show a fast positive deflection followed by a time course of variable characteristics. Three general patterns of SCC time course can be distinguished: a) Fast positive deflection of SCC (Fig. 4*A*), similar to that observed for small pulses, as already shown in Fig. 2*A*. b) Fast positive deflection of SCC followed by a marked decline with time, attaining a plateau well below the initial peak value (Fig. 4*B*). This type of time course, is seen in approximately 80% of the cases. c) Fast positive deflection of SCC followed by a marked notch and a later slow increase, reaching a plateau higher than the preceding values (Fig. 4*C*). So far, no data is available permitting correlation of the patterns displayed by the SCC time courses with known parameters of the skins.

Skins that show a small negative SCC with H_2O on their outer surfaces, normally respond to a large pulse of NaCl injected into the outer compartment with a positive deflection of SCC not preceded by the small sharp and brief negative peak. It is conceivable that the large upstroke of the SCC induced by the large NaCl pulse could mask the brief negative peak easily seen with small pulses, as shown in Fig. 2*B*.

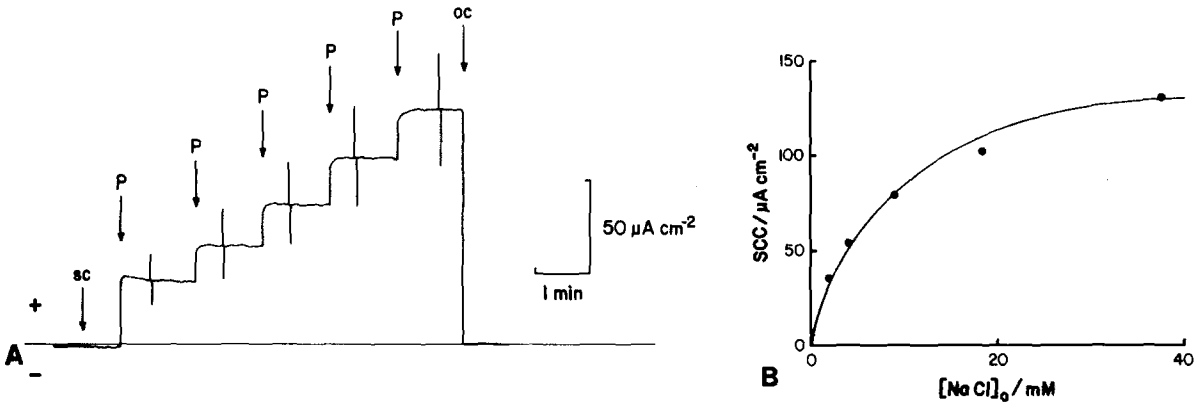

Fig. 3. (*A*) SCC time course following the injection of a series of pulses of 1 M NaCl solution into the outer compartment initially filled with H_2O, increasing $(NaCl)_o$ progressively to 2, 4, 9, 19, and 38 mM. The vertical bars are deflections of the SCC due to pulses of ± 20 mV in the clamping potential, used to monitor the skin electrical resistance. For the abbreviations *see* legend of Fig. 2. (B) Plot of SCC versus the concentration of NaCl in the outer bathing solution, $(NaCl)_o$, for the same data shown in *A*

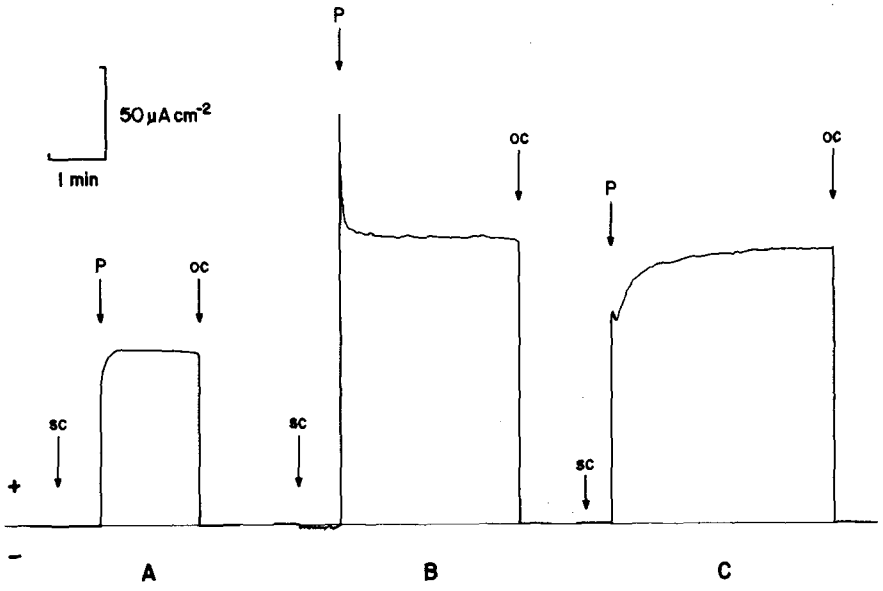

Fig. 4. SCC time course following the injection of a pulse (*P*) of 100 μl of 1 M NaCl solution into the outer compartment previously filled with H_2O, increasing $(NaCl)_o$ to 20 mM. *A*, *B*, and *C* represent three different patterns of SCC time course. In *B*, the most frequent, found in 80% of the skins is shown. For the abbreviations, *see* legend of Fig. 2

C. Effect of Amiloride

The steady-state SCC, reached after a pulse of NaCl is injected into the outer compartment, can be inhibited by a subsequent pulse of an amiloride-containing solution. This inhibitory effect depends on the amiloride concentration reached in the outer bathing solution. For a steady-state SCC of 150.4 ± 9.2 μA cm^{-2} (n = 8), attained with a $(NaCl)_o$ of 20 mM, a pulse of amiloride, increasing $(amiloride)_o$ to 6 μM, is sufficient to abolish all the amiloride-sensitive component of the SCC, as tested by a second amiloride pulse. The amiloride level of 6 μM reduces the SCC to 1.4 ± 1.3 μA cm^{-2} (n = 8). This is less than 1% of the SCC value prior to the use of the inhibitor.

Negative SCC's were never observed with the use of amiloride, even with further increases of $(amiloride)_o$ to values well above 6 μM.

Small pulses of amiloride when injected into the outer solution cause the SCC to drop in a cumulative dose-response fashion. Figure 5 shows, for a representative experiment, the effect upon the SCC of five sequential pulses of 100 μl of amiloride 10^{-4} M, which increased $(amiloride)_o$ progressively to 2, 3.8, 7.4, 15 and 22 μM.

Parallel to the fall in SCC, amiloride leads to an increase of the skin electrical resistance R. For the

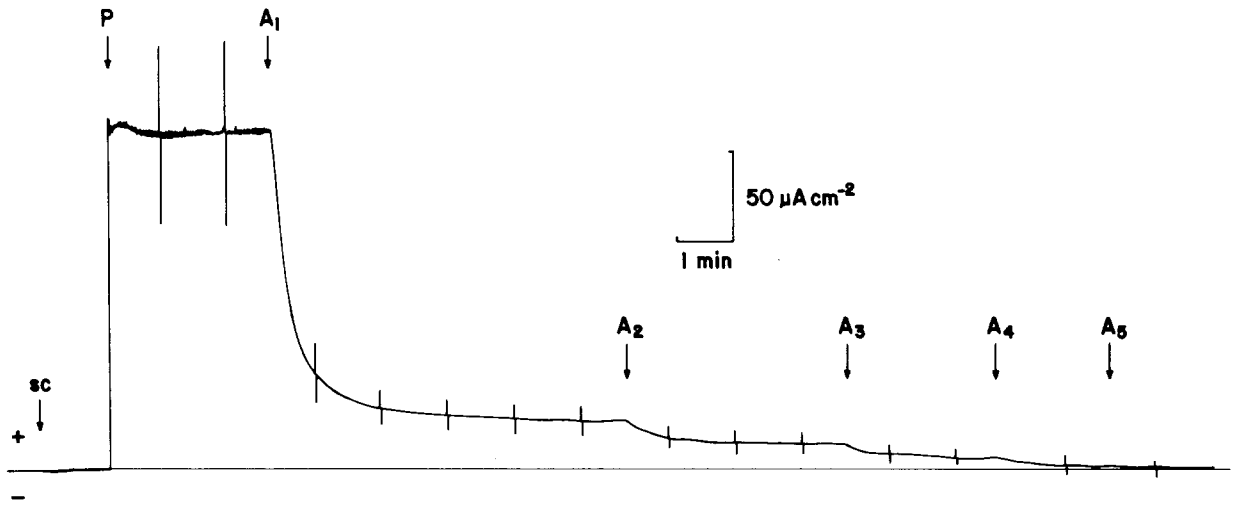

Fig. 5. SCC time course following the injection of a pulse (P) of 1 M NaCl solution into the outer compartment previously filled with H_2O, increasing $(NaCl)_o$ to 20 mM. After the SCC had stabilized, a series of pulses of amiloride (10^{-4} M) A_1, A_2, . . . A_5 were injected into the outer compartment, increasing $(amiloride)_o$ progressively to 2, 3.8, 7.4, 15 and 22 μM. The vertical bars are deflections of the SCC due to pulses of ±20 mV in the clamping potential, used to monitor the skin electrical resistance. For the abbreviations, *see* legend of Fig. 2

example shown in Fig. 5, with $(NaCl)_o$ equal to 20 mM, R was 0.56 kΩ cm^2 in the control condition and increased to a steady value of 5.23 kΩ cm^2 after amiloride had reached its full effect.

As expected from its known effects (Ehrlich & Crabbe, 1968; Nagel & Dorge, 1970; Cuthbert & Shum, 1974) the action of amiloride is reversible upon the removal of the inhibitor from the outer compartment. The reversibility was very slow when H_2O was used to rinse the outer skin surface and it took place at a much faster rate when a NaCl solution was the rinsing fluid.

To test for the specificity of Na in dislodging the amiloride molecules from their binding sites, similar experiments were performed using KCl in place of NaCl. Three groups of 10 skins each, were used. In all cases the SCC obtained with 20 mM $(NaCl)_o$ was previously and completely inhibited by 18 μM $(amiloride)_o$. After the inhibition was completed, the steady-state SCC was 1.7 ± 1.2 μA cm^{-2} (n = 30, pooled data for the three groups), the outer compartment was drained and rinsed continuously for 2 min with H_2O (group A), with 20 mM NaCl (group B) and with 20 mM KCl (group C). After that, for 30 sec, the outer compartment was thoroughly rinsed with H_2O and filled with 5 ml of H_2O. A pulse of NaCl was then injected into the outer compartment, increasing $(NaCl)_o$ to 20 mM. The SCC increased slowly in group A and at a very fast rate in groups B and C. The average SCC values obtained 10 sec after the application of the NaCl pulse were the following: 6.4 ± 2.3 μA cm^{-2} (n = 10) for group A, 76.4 ± 6.7 μA cm^{-2} (n = 10) for group B and 69.0 ± 7.1 μA cm^{-2} (n = 10) for group C. Figure 6 shows, for a single skin, the reversibility of the amiloride inhibition, as tested by a pulse of NaCl, in three different successive runs, using H_2O, 20 mM NaCl and again H_2O to rinse the outer skin surface. As can be seen, the reversibility of the amiloride inhibition is markedly larger when the salt solution was the rinsing fluid than when H_2O was used.

D. Spontaneous Decline of the Short-Circuit Current Following the Injection of a Pulse of NaCl into the Outer Compartment

As shown in Results (section B.3, Fig. 4*B*), the most frequent SCC time course seen when large pulses of NaCl were injected into the outer compartment is characterized by a sharp peak followed by a slow decline with time. Different mechanisms might contribute to the slow decline phase of the SCC.

To better understand the phenomenon responsible for the decline of SCC, the spontaneous decline of the SCC observed in the NaCl-pulse experiments was compared to the decline of SCC induced by a small dose of amiloride injected into the outer compartment. The pulse of amiloride was adequately chosen to induce a decline of SCC compara-

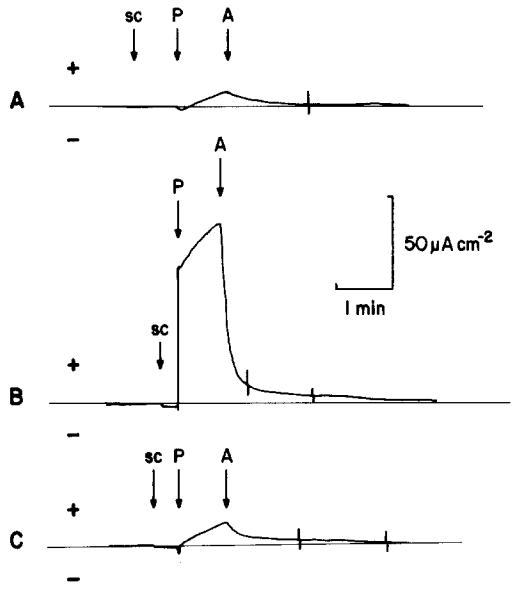

Fig. 6. Reversibility of the amiloride inhibition of the apical membrane Na channels in a single skin, as tested by a pulse of 100 μl of 1 M NaCl solution, which increased $(NaCl)_o$ to 20 mM. In each of the three successive runs the SCC was previously inhibited by amiloride (22 μM). Then the outer skin surface was thoroughly rinsed by a continuous flow of H_2O (in *A*), 0.1 M NaCl (in *B*) and again H_2O (in *C*), for 2 min. The outer compartment was then rinsed with H_2O for 30 sec and filled with 5 ml of H_2O. A pulse of 100 μl of 1 M NaCl solution was injected into the outer bathing solution, increasing $(NaCl)_o$ to 20 mM, and the SCC time course followed for approximately 1 min. Then a pulse of amiloride was injected into the outer solution, increasing $(amiloride)_o$ to 10 μM. The vertical bars are deflections of the SCC due to pulses of ±20 mV in the clamping potential, used to monitor the skin electrical resistance. For the abbreviations, *see* legend of Fig. 2

ble to the spontaneous decline of SCC observed in the NaCl-pulse experiments. The skin electrical resistance (R) was measured along the spontaneous decline of SCC, in the NaCl-pulse experiments, and along the decline of SCC induced by amiloride. The rationale of these experiments assumes that an increase in R is expected to occur along the spontaneous decline of the SCC, in the NaCl-pulse experiments, if a progressive block of the apical membrane Na channels occurs. In contrast, if the decline of the SCC were due to compartment filling, that is, Na accumulation within the apical cells, reducing the driving force for Na electrodiffusion across the apical Na channels, no change or even a decrease in R would be expected, since the single-channel conductance of ionic channels are expected to be a function of the concentration of the permeating species in the solutions bathing their ends, as exemplified by the gramicidin A channel (Finkelstein & Andersen, 1981). Figure 7 shows, for a representative experiment the spontaneous decline of the SCC following a NaCl pulse injected into the outer compartment and the decline of the SCC induced by a pulse of amiloride also injected into the same compartment. In a group of nine skins studied with the same protocol, R decreased from 0.61 ± 0.08 to 0.49 ± 0.08 kΩ cm^2 during the spontaneous decline of SCC and increased from 0.49 ± 0.08 to 0.92 ± 0.07 kΩ cm^2 under the effect of amiloride. As seen, along the spontaneous decline of the SCC a small but consistent decline of R was observed. On the other hand, the decline of the SCC induced by amiloride was accompanied by a marked increase of this electrical parameter. These results rule out the closure of the apical membrane Na channels as the sole factor responsible for the spontaneous decline of the SCC, and strongly suggest that the spontaneous decline is mostly due to compartment filling, with a consequent reduction of the driving force for the inward movement of Na into the epithelial cells.

Discussion

The chamber specially developed for this study permitted me to follow the SCC and other skin parameters, as PD and R, induced by a step increase of solute (salts or inhibitors) concentration in the outer bathing medium, vigorously stirred to minimize the effects of the unstirred layer adjacent to the outer skin surface. Unstirred layers in biological membranes have been considered previously (Kidder et al., 1964; Dainty & House, 1966; Fuchs et al., 1972; Carmona & Lacaz-Vieira, 1979). The vigorous stirring aimed also to induce a fast equilibrium of the concentration when pulses of concentrated solutions were injected into the outer bathing medium. As compared to the slow time course of the SCC transients seen in the present study, a half-time of 18 msec for the outer bathing solution to reach equilibrium, when salt pulses were given, indicates that delays in mixing do not distort the recorded time course of the SCC transients.

Short-circuiting the skin with outer bathing solutions of low electrical conductivity pose some technical difficulties. We opted to reduce to a minimum the electrical resistance between the voltage-sensing bridges and the skin surfaces, rather than using automatic compensation for the voltage drop between these two regions, because of the large differences in conductivities of solutions bathing the skin surfaces, and also due to the large and fast changes of the electrical conductivity of the outer solution following the injection of pulses of NaCl into this medium.

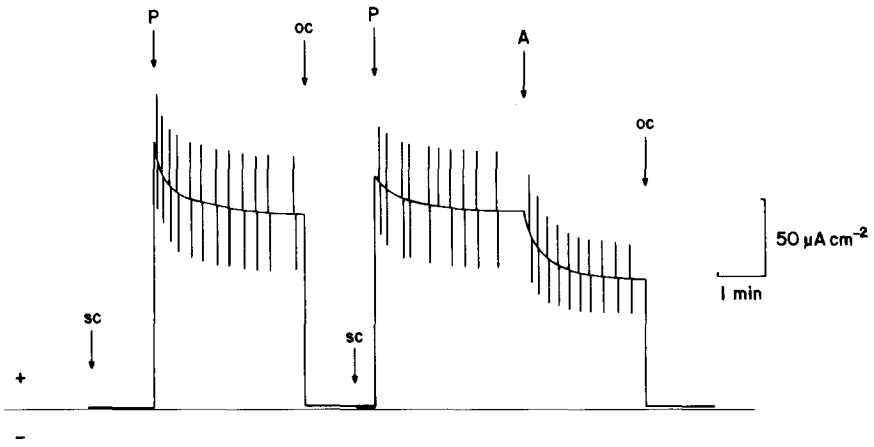

Fig. 7. Comparison of the spontaneous decline of the SCC with that induced by an adequate pulse of amiloride to induce a decline in the SCC similar to that seen spontaneously after a pulse of NaCl was injected into the outer compartment. From left to right: The first run is the SCC time course following the injection of 100 μl of 1 M NaCl solution into the outer bathing solution, increasing $(NaCl)_o$ to 20 mM. The second run is similar to the first. Then, in *A* a pulse of 5 μl of an amiloride solution (10^{-4} M), increasing $(amiloride)_o$ to 0.1 μM, was applied to the outer compartment. The vertical bars are deflections of the SCC due to pulses of ±20 mV in the clamping potential, used to monitor the skin electrical resistance. For the abbreviations, *see* legend of Fig. 2

A priori, the SCC could be due not only to the transepithelial active Na transport (Ussing & Zerahn, 1951), but also to the passive movement of Na, Cl and other ions across the skin along their electrochemical gradients, as solutions for very different compositions bathe each side of the skin. The experiments with amiloride, in which the SCC was virtually abolished by 6 μM $(amiloride)_o$, strongly supports that the SCC is a fair measurement of the net Na movement across the apical membrane Na channels. This, however, cannot be taken as a definite indication that in the short-circuit condition Na is the only ion moving across the skin. It is conceivable that salt leaks could take place through different routes across the skin, the tight junctions being among the most plausible pathways. Ion flows in pathways which do not discriminate between cations and anions are not expected to contribute to the clamping current in the short-circuited state. In very leaky skins (Results, section A) the SCC observed with H_2O as outer bathing fluid, which increased with time and could be abolished by a continuous flow of H_2O across the outer compartment, is a strong indication for the existence in these skins of an important electrically silent salt leak, probably NaCl, from the skin to the outer compartment. The negative SCC could, in principle, result from differences between anion and cation mobilities in the leak pathways. These leaky skins were discarded and the study which follows was performed in preparations showing a null or, in a few cases, a very small but stable negative SCC with H_2O as outer bathing fluid.

The SCC time couse inducted by small increases of $(NaCl)_o$ (Fig. 2*A*) is compatible with a passive behavior of the apical membrane, excluding the participation of relatively slow regulatory mechanisms operating upon the apical membrane Na permeability. Those skins displaying a small and stable negative SCC with H_2O as the outer bathing fluid respond to small pulses of NaCl with a negative SCC peak preceding the positive step (Fig. 2*B*). The nature of the transient ionic currents which contribute to this peak is so far unknown. Regulatory mechanisms operating upon the apical Na permeability are neither evident when the SCC is increased to large values by the application of a series of small pulses or of pulses of increasing magnitude. The SCC increases in a staircase fashion (Fig. 3*A*) and the relationship between SCC and $(NaCl)_o$ shows a tendency to saturation (Fig. 3*B*) resembling very closely the hyperbolic relationship between SCC and $(Na)_o$ in experiments where the ionic strength of the outer solution was kept constant and equal to that of the Ringer's solution (Ussing, 1949; Kirschner, 1955; Cereijido et al., 1964).

When large pulses of NaCl were used, in most of the experiments increasing $(NaCl)_o$ to 20 mM, the SCC showed a time-course variable with the intrinsic condition of each skin. For a small percentage of preparations, the SCC time evolution was similar to that seen when small pulses of NaCl were used, except for being of larger amplitude (Fig. 4*A*). In another small group of skins, the SCC showed a biphasic response (Fig. 4*C*) which might result from two distinct mechanisms, which partially overlap

and affect the Na movement. A faster one, responsible for the decline of the SCC in the descending part of the notch is assumed to be due to the same basic process which induces the decline of the SCC that will be discussed next. A slower one, causing the late increase of the SCC might reflect a slow activation of the basolateral membrane Na pump.

The majority (80%) of the preparations responds to a large pulse of NaCl with a sharp rise of the SCC followed by a slow decline with time (Fig. 4*B*) which, in principle, could result from different processes. Na self-inhibition (Erlij & Smith, 1973; Zeiske & Lindemann, 1974; Bevevino & Lacaz-Vieira, 1982; Van Driessche & Erlij, 1983; Lindemann, 1984) or feedback inhibition (Cereijido et al., 1964, Fuchs et al., 1977) of the apical membrane Na channels could lead to a decline of the SCC as a consequence of a reduction of the Na permeability of the outer barrier. A similar effect could result from Na accumulation in the cytoplasm of the apical cells (Morel & Leblanc 1973, 1975), reducing the driving force for Na electrodiffusion into these cells. The comparison of the skin electrical resistance (R) along the spontaneous decline of the SCC in the NaCl-pulse experiments, in which R remained constant in some skins but, on the average, showed a small decrease, with the decline induced by amiloride, where R increased markedly, indicates that reduction of the apical membrane Na permeability cannot be taken as the sole factor responsible for the spontaneous decline of the SCC in the NaCl-pulse experiments (Fig. 4*B*). Reduction of the driving force for Na across the apical membrane due to an increase of $(Na)_{cell}$ remains a strong alternative explanation for the decline of the SCC. Increase of $(Na)_{cell}$ would not only cause a reduction of the Na flow across the apical Na channels but also a drop of R, as the conductance of sodium channel is expected to be a function of the channel permeability and the Na concentrations in the solutions bathing the Na channel ends. An increase of $(Na)_{cell}$ would be expected to increase the Na conductance of the apical membrane leading to a decline of R. Similar behavior was described for the gramicidin A channel (Finkelstein & Andersen, 1981) and for the toad bladder (Palmer, 1985) where an increase of the Na conductance of the apical membrane, not accompanied by an increase of $(P^{Na})_o$ was observed under the action of ouabain, which increases the cell Na concentration. The interpretation for the spontaneous decline of SCC due to increase of $(Na)_{cell}$, in spite of being a plausible one, is not unique. A drop of $(P^{Na})_o$ by self-inhibition or feedback inhibition of the Na channels associated to a large increase of ion conductances in other regions of the epithelium cannot be discarded. We have no evidence whatsoever for increases in ion conductances in the epithelium. Rough calculations based on the changes in SCC and time course under the assumption that the changes in $(Na)_{cell}$ take place mostly in the outermost living cell layer of the epithelium indicate that enough Na actually enters the cells during the decline of SCC to reduce the Na gradient.

Large changes in $(Na)_{cell}$ following a step increase of $(NaCl)_o$, are expected to occur in epithelial cells having a high apical membrane Na permeability and a Na pump of high affinity but of low transport capacity. These cells would have a very low Na concentration in the absence of NaCl on their apical surfaces and a large increase of $(Na)_{cell}$ would result from a sudden rise of $(NaCl)_o$. Frog skins in steady-state condition appear to have highly coupled epithelial cells, except for the mitochondria-rich cells and the glandular epithelium (Rick et al., 1978; Dorge et al., 1979). In our case, however, where transients are studied, the degree of cell-to-cell communication plays a fundamental role in determining whether all cells or only the most superficial ones would show fluctuations of $(Na)_{cell}$ in response to a step increase of $(NaCl)_o$.

It is worth comparing the spontaneous decline of SCC observed in the NaCl pulse experiments, where $(Na)_o$ and $(Cl)_o$ increase suddenly and together, with the decline of PD observed in the experiments of Lindemann and Gebhardt (1973), where the step increase of $(Na)_o$ was associated with an equivalent reduction of $(K)_o$. According to these authors, the decline of PD could not be explained by a reduction of the Na driving force due to an increase of $(Na)_{cell}$. One of their arguments in favor of this hypothesis was the progressive increase of the skin electrical resistance which took place concomitantly with the decline of PD. Despite the mentioned differences in protocol, our results are in conflict with theirs.

Various parameters that influence the SCC time course are still obscure and more data need to be gathered to permit a clear picture of the phenomenon.

The last point to be addressed deals with the reversibility of the amiloride effect. According to the current views expressed in the literature, the Na channels in the apical membrane of Na transporting epithelia have binding sites and regulatory sites which are implicated in the Na-entry step. In toad bladder it has been suggested that a carboxyl group in an amphipatic phase of the apical membrane may be involved in the passage of Na through the channel (Park et al., 1983). Likewise, a similar functional group with pK_a of 4 to 5 was implicated in the Na-

entry step in the frog skin (Zeiske & Lindemann, 1975; Cuthbert, 1976). Amiloride must interact with at least two sites on the Na channel in order to accomplish its inhibitory effect (Park & Fanestil, 1983). The electrostatic interaction between the cationic guanidinium group of the amiloride molecule and the anionic carboxyl residue in the channel structure is apparently the first step in the process of amiloride blocking (Fanestil et al., 1984). We have shown (Results, section C, Fig. 6) that the reversibility of the amiloride effect is highly dependent upon the ionic strength of the solution used to rinse the outer skin surface. When H_2O is used, the amiloride molecules washout slowly from their binding sites as judged from the slow reversal of its inhibitory effect. On the other hand, when NaCl or KCl solutions replace H_2O as the rinsing solution, the amiloride washout was speeded up markedly. As no significant difference was seen between the inhibition recovery process when NaCl or KCl solutions were used, it may be concluded that there is no specificity for Na ions in removing the amiloride molecule from its binding site and that this effect should be of a purely electrostatic nature. So, the carboxyl group in the Na channel would work as an anionic site of cation-exchange resin which binds firmly to the cationic guanidinium moiety of the amiloride molecule, which could only be replaced by exchange with another positively charged species, like Na or K ions.

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