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Control of Sodium Permeability of the Outer Barrier in Toad Skin

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Summary. The ^{24}Na efflux (J_{eff}^{Na}) (i.e., the rate of appearance of ^{24}Na in the outer compartment) in the isolated short-circuited toad skin bathed by NaCl-Ringer's solution on both sides is composed of para- and transcellular components of almost equal magnitudes. This relies on the assumption that amiloride acts on the transcellular component only and could block it completely.

Ouabain induces a large transient increase of the transcellular component. This increase, which starts within a few minutes after the addition of ouabain, is due to electrical depolarization of the outer barrier, rather than a consequence of blocking Na recirculation across the inner barrier. The subsequent decline of J_{eff}^{Na}, which takes place after the ouabain-induced J_{eff}^{Na} peak, is due to a progressive block of outer barrier Na channels with time, which can eventually be complete, depending on the duration of action of ouabain. As the external Na concentration was always kept high and constant in these experiments, the results indicate that a rise in cell Na concentration, and not in the outer bathing solution, is the signal that triggers the reduction of outer barrier Na permeability (P_o^{Na}).

Ouabain has no effect upon J_{eff}^{Na} with Na-free solution bathing the outer and NaCl-Ringer's solution the inner skin surface, showing the importance of Na penetration across the outer barrier, and not across the inner barrier due to its low Na permeability, in the process of closing the Na channels of this structure.

Step changes from Na 115 mM to Na-free external solution, or vice-versa, may affect both the outer barrier electrical potential difference (PD_o) and cell Na concentration ($Na)_c$. Therefore, the behavior of J_{eff}^{Na} depends on which variable (if PD_o or $(Na)_c$ regulated outer barrier Na permeability) is most affected by step changes in outer bathing solution Na concentration.

Amiloride in the control condition blocks the transcellular component of J_{eff}^{Na}. However, in the condition of approximate short-circuiting of the outer barrier and high cellular Na concentration induced by long term effects of ouabain, when the Na channels of the outer barrier are already blocked by elevated cell Na concentration, amiloride may induce the opposite effect, increasing Na permeability of the outer barrier.

With outer barrier Na channels completely blocked by high cell Na concentration, PCMB in the outer bathing medium induces a large increase of J_{eff}^{Na}, rendering these channels again amiloride sensitive.

The results are consistent with the notion that Na efflux from cell compartment to the outer bathing solution goes through the amiloride-sensitive Na channels of the apical border of the superficial cell layer of toad skin, with an apparent Na permeability modulated by cell ionic environment, most probably the cell Na concentration.

The ensemble of the present results are consistent with Na permeability regulation taking place at the outer barrier level. However, this precise location could only be made unambiguously by measurements across the individual outer cell membranes.

Key words toad skin · sodium permeability · permeability regulation · ouabain · amiloride · sodium channels

Introduction

The model of two barriers in series for the Na transport across amphibian skin (Koefoed-Johnsen & Ussing, 1958) assumes that the penetration of Na ions across the outer barrier is a simple diffusion process. Cereijido, Herrera, Flanigan & Curran (1964) suggested that the "apparent Na permeability" of the outer barrier of skin, measured through the evaluation of rate coefficients for unidirectional Na movement, decreases markedly when Na concentration in the outer solution is raised. They suggested that the movement of Na across the outer membrane might not be entirely due to simple diffusion and postulated that it could be a facilitated diffusion. In 1973, Erlij and Smith showed that ouabain inhibits Na uptake across the outer barrier only in the presence of Na ions in the bathing solutions and concluded that ouabain inhibition of Na uptake is mediated primarily through an increase of epithelial Na concentration. Biber (1971), Moreno et al. (1973) and Leblanc and Morel (1975) also reported results consistent with cellular Na concentration regulating the Na uptake mechanism across the outer membrane of amphibian skin. On the other hand, Fuchs, Hviid-Larsen and Lindeman (1977) present contrasting evidence that the Na concentration in the outer bathing solution, $(Na)_o$, and not the cellular Na concentration, $(Na)_c$, is the variable controlling the Na permeability of the outer barrier, (P_o^{Na}): at constant $(Na)_c$, the steady-state value of $1/P_o^{Na}$ increases linearly with $(Na)_o$. They concluded that the transport through the outward-facing

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membrane of the *stratum granulosum* cells can be described as an electrodiffusion process which, as such, does not saturate with increasing $(Na)_o$. However, when added to the outer border of the apical membrane, Na causes a decrease of P_o^{Na} within several seconds, which they interpreted as due to Na binding inducing closure of the Na channels. A similar conclusion was obtained by Van Driessche and Lindemann (1979) on the ground of noise analysis, concluding that the density of conducting pores decreases with increasing $(Na)_o$.

There are also in the literature controversial viewpoints for different epithelial membranes, regarding the role of cell ionic environment or, more particularly, the cell Na concentration (Lewis & Diamond, 1976; Cuthbert & Shum, 1978; Shum & Fanelli, 1978; Turnheim et al. 1978; Aceves & Cuthbert, 1979; Chase & Al-Aqati, 1979; Helman, Nagel & Fisher, 1979), or the cytosolic calcium concentration (Balaban & Mandel, 1979; Taylor & Windhager, 1979) on the regulation of Na permeability of the outer barrier.

The purpose of the present study was to examine further the relation between changes in outer barrier Na permeability and maneuvers altering the outer or the cytosolic Na concentration.

Some of the results have been previously reported at the International Congress of Physiological Sciences (Lacaz-Vieira & Bevevino, 1980) and the Meeting of the Brazilian Academy of Sciences (Bevevino & Lacaz-Vieira, 1980).

Materials and Methods

These studies were carried out in modified Ussing-Zerahn chambers, according to methods previously described (Varanda & Lacaz-Vieira, 1978, 1979). Special precautions were taken to prevent the effect of skin edge damage (Helman & Miller, 1971, 1973, 1974; Biber & Mullen, 1977) on the low levels of Na efflux by using hemichambers provided with a circular groove (4 mm wide and 0.4 mm deep filled with silicone grease – Dow Corning High Vacuum Grease) located at the internal rim of the hemichamber surface in contact with the epithelial side of the skin (Varanda & Lacaz-Vieira, 1978). This mounting procedure very efficiently prevents the contact of the damaged area with the bathing solutions. Abdominal skins of the toad *Bufo marinus ictericus* were used and the experiments performed in the short-circuited state at room temperature (20 to 25 °C). A voltage-clamp unit was connected to the preparation through 3 M KCl agar bridges and saturated KCl calomel half-cells (for voltage measurements) and Cu-$CuSO_4$ half-cells (for current passing). An equilibration period of approximately 1 hr or more, according to the protocol of each experimental group (as will be referred in Results) elapsed before the addition of approximately 100 μCi of ^{24}Na (Institute of Atomic Energy, São Paulo, Brazil) to the solution bathing the inner skin surface (corial side). Every 2 to 5 min, according to the experimental protocol, all the solution bathing the outer skin surface was totally drained into counting vials for ^{24}Na assay in an automatic gamma counter (Nuclear Chicago, mod. 4230). Short-circuited condition was maintained throughout the experiments, except for 5 to 10 sec during drainage of the outer compartment. In the kinetic experiments, collection from the outer compartment started immediately following addition of the isotope to the inner compartment. In others, collection started after an equilibration period, long enough for the Na efflux across the skin to attain a stationary state. J_{eff}^{Na} is the Na efflux from epithelium to the outer bathing solution, calculated from the rate of appearance of ^{24}Na in the outer compartment.

Solutions used were: NaCl-Ringer's solution (in mM): NaCl, 115; $KHCO_3$, 2.5; $CaCl_2$, 1.0. Na_2SO_4-Ringer's solution (in mM): Na_2SO_4, 57.5; $KHCO_3$, 2.5; $CaSO_4$, 1.0. KCl- or K_2SO_4-Ringer's solution were obtained by equimolar substitution of Na by K in the NaCl- or in the Na_2SO_4-Ringer's solution, respectively. All the solutions had pH 8.2 after aeration. Drugs used were: Ouabain and *p*-chloromercuribenzoate (sodium salt) from Sigma Chemical Company and amiloride from Merck Sharp & Dohme Research Laboratories. Solubilization of *p*-chloromercuribenzoate was obtained by dissolving the desired amount of drug in approximately 5 ml of Ringer's solution made alkaline with KOH or NaOH to pH 9.5, the volume completed to 500 ml and the pH adjusted back to 8.2 with HCl or H_2SO_4.

Results are presented as mean ± standard error, and n is the number experiments.

Results

1. Effect of Amiloride on J_{eff}^{Na} in Short-Circuited Skins Bathed on Both Sides by NaCl-Ringer's Solution

These experiments were performed with NaCl-Ringer's solution bathing both sides of skin. After J_{eff}^{Na} had reached a steady state, normally within 30 min after addition of the isotope to the inner compartment, NaCl-Ringer's solution containing amiloride (10^{-4} M) was used to bathe the outer skin surface. Immediately a sharp reduction was observed in J_{eff}^{Na} and in SCC. SCC fell to less than 2% of its initial value in 10 min. In the steady state before amiloride, J_{eff}^{Na} was equal to 1.24 ± 0.17 nmol cm^{-2} min^{-1} and after amiloride, equal to 0.70 ± 0.12 nmol cm^{-2} min^{-1}, indicating a reduction of 44% ($P < 0.01$, paired t test, $n = 9$). These results are an indication that in the present experimental condition, almost half of the Na efflux goes across the skin through a transcellular route and the rest, via a paracellular pathway.

2. Effect of Ouabain and Subsequent Action of Amiloride on J_{eff}^{Na} in Short-Circuited Skins Bathed by NaCl-Ringer's Solution on the Inner Side

2.1. Experiments Performed with NaCl-Ringer's Solution Bathing the Outer Skin Surface. After J_{eff}^{Na} had reached steady state following addition of the isotope to the inner solution, with J_{eff}^{Na} equal to 1.07 ± 0.22 nmol cm^{-2} min^{-1}, ouabain was added to the inner solution to a concentration of 10^{-3} M. Soon after oua-

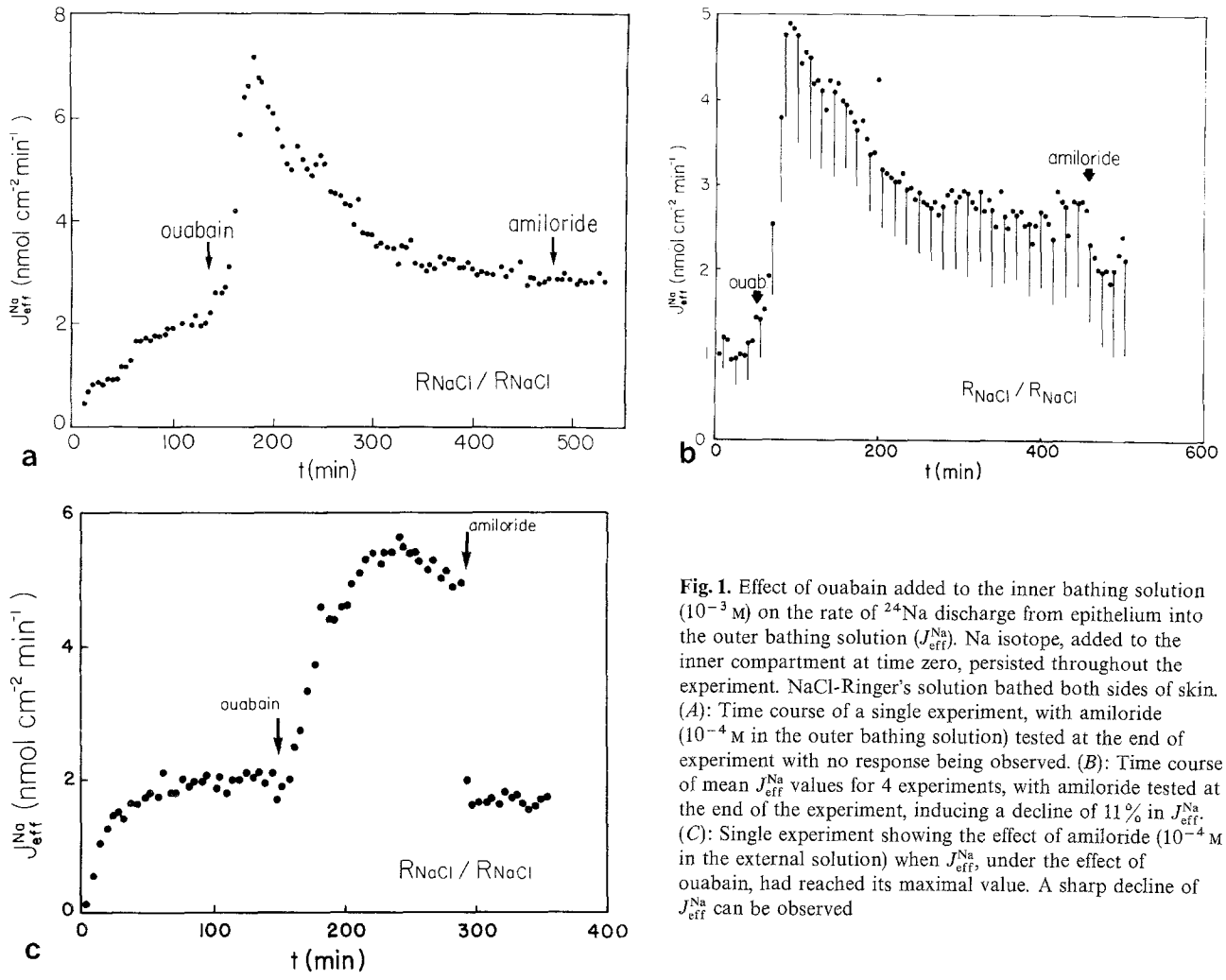

Fig. 1. Effect of ouabain added to the inner bathing solution (10^{-3} M) on the rate of ^{24}Na discharge from epithelium into the outer bathing solution (J_{eff}^{Na}). Na isotope, added to the inner compartment at time zero, persisted throughout the experiment. NaCl-Ringer's solution bathed both sides of skin. (*A*): Time course of a single experiment, with amiloride (10^{-4} M in the outer bathing solution) tested at the end of experiment with no response being observed. (*B*): Time course of mean J_{eff}^{Na} values for 4 experiments, with amiloride tested at the end of the experiment, inducing a decline of 11% in J_{eff}^{Na}. (*C*): Single experiment showing the effect of amiloride (10^{-4} M in the external solution) when J_{eff}^{Na}, under the effect of ouabain, had reached its maximal value. A sharp decline of J_{eff}^{Na} can be observed

bain, J_{eff}^{Na} displayed a transient increase with time to reach a peak of 3.89 ± 0.52 nmol cm^{-2} min^{-1} (285% above the control stationary value prior to ouabain) ($P < 0.01$, paired t test, $n = 18$), 62 ± 9 min after the inhibitor was introduced in the inner compartment. Following the peak, J_{eff}^{Na} declined with time to a new steady state which was reached approximately 250 min after ouabain was introduced in the system. Figure 1 shows a transient behavior of a single experiment after ouabain was added to the inner solution. Under the effect of ouabain, SCC declined progressively with time to reach approximately 8% of its initial value after 1 hr and less than 1% after 2 hr.

The effect of amiloride (10^{-4} M) in the outer solution upon J_{eff}^{Na} was tested at two different times after ouabain addition: (i) in the stationary state and (ii) in the region of the peak.

Figure 1*b* shows the effect of amiloride on J_{eff}^{Na} stationary state attained after ouabain. A small reduction of 11%, though statistically significant ($P = 0.04$, paired t test, $n = 4$) was observed on J_{eff}^{Na}, which fell from 2.37 ± 0.96 nmol cm^{-2} min^{-1} to 2.10 ± 0.94 nmol cm^{-2} min^{-1}. Figure 1*a* shows the behavior of a single experiment where amiloride had no effect whatsoever upon J_{eff}^{Na}.

In contrast, amiloride had a profound and sharp effect upon J_{eff}^{Na} when tested at the peak induced by ouabain, as shown in Fig. 1*c*, for a single experiment. The presence of amiloride in the outer solution reduced J_{eff}^{Na} from a peak value of 4.40 ± 0.90 nmol cm^{-2} min^{-1}, obtained prior to amiloride, to 1.75 ± 0.30 nmol cm^{-2} min^{-1}, a reduction of 60% in J_{eff}^{Na} ($P < 0.01$, paired t test, $n = 5$).

The fact that amiloride has a much larger effect upon J_{eff}^{Na} when tested at the peak induced by ouabain than in the steady state after ouabain, indicates that the transient increase of J_{eff}^{Na} is certainly due to a transient increase of the transcellular component of J_{eff}^{Na} and not of the paracellular fraction of J_{eff}^{Na}.

It is interesting to notice that amiloride in the control condition without ouabain (Section 1) reduces J_{eff}^{Na} to a new level, 44% below the control steady

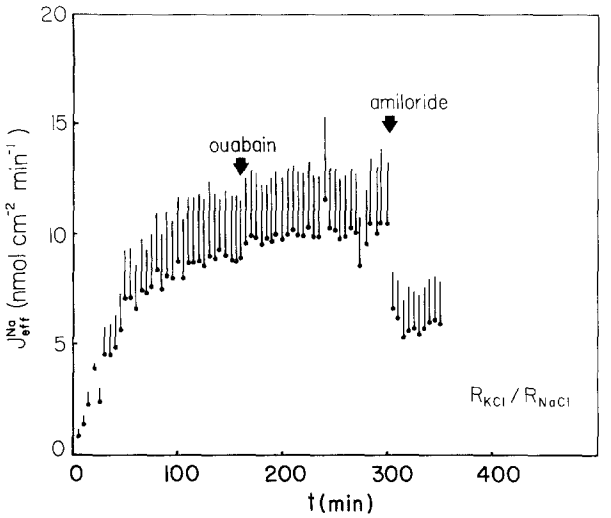

Fig. 2. Effect of ouabain added to the inner bathing solution (10^{-3} M) on the rate of ^{24}Na discharge from epithelium into the outer bathing solution (J^{Na}_{eff}). Skins were bathed by NaCl-Ringer's solution on the inner side and KCl-Ringer's solution on the outer side. Na isotope was added to the inner compartment at time zero and persisted throughout the experiment. Ouabain was tested when J^{Na}_{eff} had reached a steady state, and no detectable effect upon J^{Na}_{eff} can be observed. Subsequently, amiloride (10^{-4} M) in KCl-Ringer's solution was used to bathe the outer skin surface, reducing J^{Na}_{eff} of 42% ($n=7$)

state. However, after ouabain, the effect of amiloride depends on the length of time ouabain was in contact with the skin. Thus, Fig. 1*c* shows that in the J^{Na}_{eff} peak induced by ouabain, amiloride reduces J^{Na}_{eff} to a value approximately equal to the control steady-state value prior to the action of ouabain. On the other hand, in the steady state after ouabain, the effect of amiloride is null (Fig. 1*a*) or very small (Fig. 1*b*). In this new steady-state condition, J^{Na}_{eff} value is almost double that of the control steady state before ouabain.

2.2. Experiments Performed with KCl-Ringer's Solution Bathing the Outer Skin Surface. Figure 2 shows the time course of J^{Na}_{eff} after the Na isotope was added to the inner bathing solution in experiments performed with KCl-Ringer's solution bathing the outer skin surface for at least 2 hr before addition of Na isotope. When a quasi-J^{Na}_{eff}-steady-state was reached, ouabain was added to the inner solution to a concentration of 10^{-3} M, and no effect, whatsoever, was observed upon J^{Na}_{eff}. This behavior is in a clear contrast with that displayed by J^{Na}_{eff} when Na ions were present in the outer bathing solution (Section 2.1). This preparation with K replacing Na in the outer bathing solution, which is insensitive to ouabain, is, however, still very sensitive to amiloride (10^{-4} M) in the outer medium, as shown in Fig. 2.

3. Effect of Na-free Outer Bathing Solution on J^{Na}_{eff} in Short-Circuited Skins Bathed by NaCl-Ringer's Solution on Both Sides

Skins were bathed by NaCl-Ringer's solution on both sides. After the rate of ^{24}Na discharge to the outer bathing solution had reached a stationary state, all the Na in that solution was substituted by K on equimolar basis. In the steady state, with Na in the outer bathing solution, J^{Na}_{eff} was equal to 1.17 ± 0.10 nmol cm^{-2} min^{-1}, and after the ionic substitution, a new steady state was reached almost immediately with J^{Na}_{eff} equal to 0.90 ± 0.06 nmol cm^{-2} min^{-1}, indicating a reduction of 23% in J^{Na}_{eff} ($P=0.01$, paired t test, $n=20$).

4. Effect of Na-Free Outer Bathing Solution on J^{Na}_{eff} in Short-Circuited Ouabain-Treated Skins Bathed by NaCl-Ringer's Solution on Both Sides

After J^{Na}_{eff} had reached a stationary level with NaCl-Ringer's solution bathing both sides of skin, ouabain was added to the inner compartment and a rise was observed in J^{Na}_{eff}, as already described in Section 2.1. When J^{Na}_{eff} had reached the peak induced by ouabain, all the Na in the outer bathing solution was substituted by K on equimolar basis. Contrary to what was observed in the previous section, under the present experimental condition, a sharp increase followed by a slow decline with time was observed in J^{Na}_{eff} after Na was substituted by K in the outer compartment, as shown in Fig. 3. Prior to the ionic substitution, J^{Na}_{eff} was equal to 2.00 ± 0.32 nmol cm^{-2} min^{-1}, and immediately following the ionic substitution, J^{Na}_{eff} increased to 3.87 ± 0.56 nmol cm^{-2} min^{-1} (mean of the J^{Na}_{eff} peak values observed after the ionic substitution, normally the second value in the time sequence). This value is significantly higher (91% increase) than that observed prior to the substitution of Na by K in the external compartment ($P<0.01$, paired t test, $n=6$). After that increase, J^{Na}_{eff} declined to a new steady state equal to 2.53 ± 0.21 nmol cm^{-2} min^{-1}, that is still above the control value with Na in the outer compartment.

5. Effect of Amiloride on J^{Na}_{eff} under the Condition of High Cell Na Concentration and Approximate Short Circuiting of the Outer Barrier

These experiments were performed with skins equilibrated for at least 3 hr in Na_2SO_4-Ringer's solution in the outer compartment and K_2SO_4-Ringer's solution plus ouabain (10^{-3} M) in the inner compartment. High inner K concentration was used to depolarize the inner barrier and increase its electrical conduc-

tance in order to permit on approximate voltage-clamp condition of the outer barrier when the whole skin is clamped (Leblanc & Morel, 1975; Morel & Leblanc, 1975; Fuchs et al., 1977). Ouabain was used to eliminate a significant electrogenic component of the Na pump which has been shown to be present in the skin of the toad *Bufo marinus ictericus* (Lacaz-Vieira, Varanda, Bevevino & Fernandes, 1979; Varanda & Lacaz-Vieira, 1979). After this equilibration period, ^{24}Na was added to the inner compartment and J_{eff}^{Na} followed until an approximate steady state was attained. Then, Na_2SO_4-Ringer's solution containing amiloride (10^{-4} M) was used to bathe the outer skin surface. In five skins, no effect whatsoever was observed in J_{eff}^{Na} under the action of amiloride (Fig. 4*a*). In seven other skins, amiloride had an effect opposite to that which would be expected from its known inhibitory effect on Na conductance of the outer barrier, inducing a stimulatory effect upon J_{eff}^{Na}, which increased under the effect of this drug, as shown in Fig. 4*b* for four representative experiments.

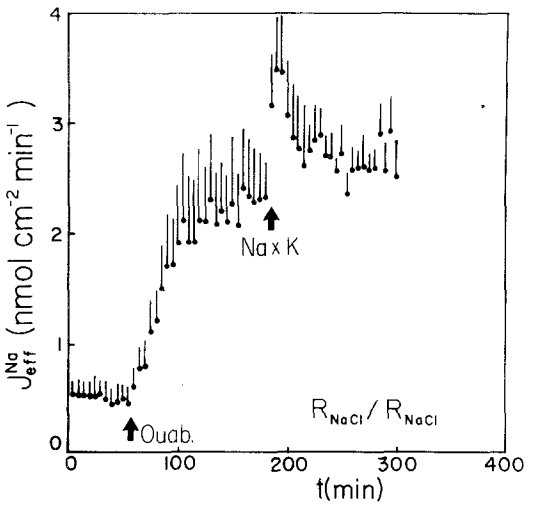

Fig. 3. Effect of Na by K substitution in the outer bathing solution on the steady-state rate of ^{24}Na discharge from epithelium into the outer bathing solution (J_{eff}^{Na}) in short-circuited ouabain-treated skins bathed by NaCl-Ringer's solution on both sides. Ouabain was used in a concentration of 1 mM in the inner bathing solution. Na was substituted by K on equimolar basis in the outer bathing solution, ($n=6$)

6. *Effect of PCMB on J_{eff}^{Na} in Short-Circuited Skins Bathed on Both Sides by Na_2SO_4-Ringer's Solution, with High Cell Na Concentration Induced by Long-Term Effect of Ouabain*

These experiments were performed in skins equilibrated for at least 3 hr with Na_2SO_4-Ringer's solution on both sides, plus ouabain (10^{-3} M) in the inner solution. After this equilibration period, ^{24}Na was added to the inner compartment. When J_{eff}^{Na} steady state was reached, the outer bathing solution was changed to Na_2SO_4-Ringer's solution containing *p*-chloromercuribenzoate (PCMB) at a concentration of 1 mM. The contact of PCMB with the outer skin surface induced a large increase in J_{eff}^{Na}, of the order of 110%, from a steady-state value equal to 2.07 ± 0.04 nmol cm^{-2} min^{-1} to a new steady-state level of 4.35 ± 0.15 nmol cm^{-2} min^{-1} ($P<0.01$, paired *t* test, $n=6$), as shown in Fig. 5 as normalized

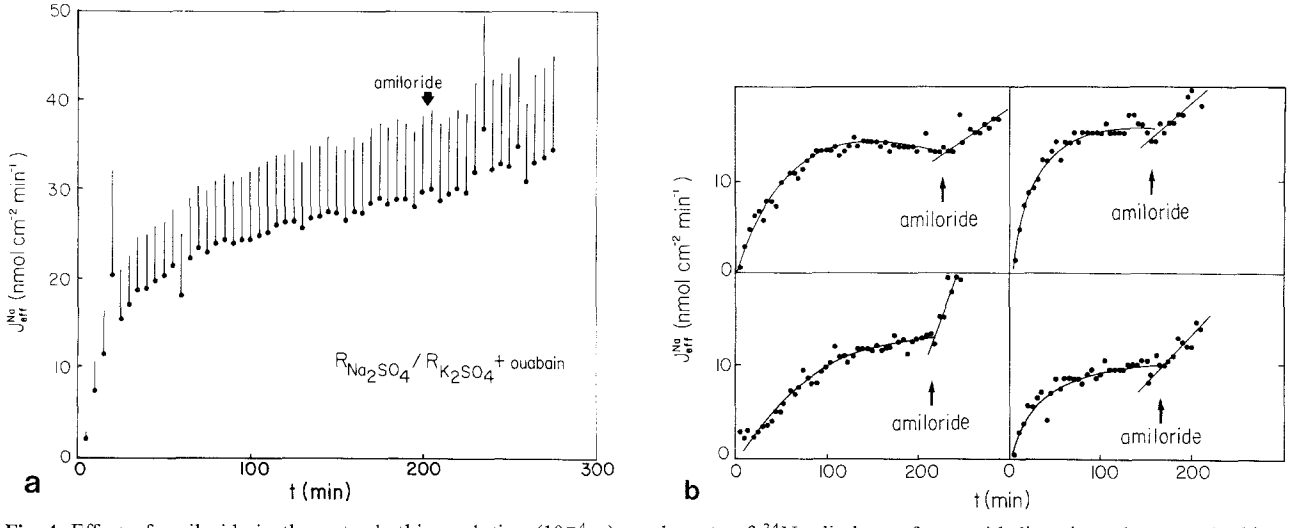

Fig. 4. Effect of amiloride in the outer bathing solution (10^{-4} M) on the rate of ^{24}Na discharge from epithelium into the outer bathing solution (J_{eff}^{Na}) under the condition of high cell Na concentration and approximate short circuiting of the outer barrier. The bathing media were: external solution; Na_2SO_4-Ringer's solution; internal solution; K_2SO_4-Ringer's solution plus ouabain 1 mM. Na isotope was added to the inner compartment at time zero. (*A*): Mean values of six experiments where amiloride had no effect upon J_{eff}^{Na}. (*B*): Four single experiments where amiloride induced an increase of J_{eff}^{Na} immediately following its addition to the outer compartment

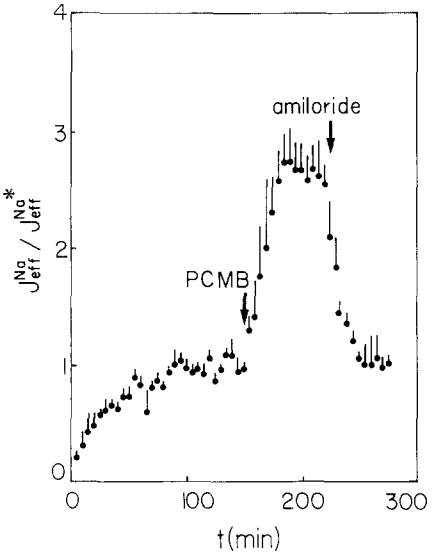

Fig. 5. Effect of *p*-chloromercuribenzoate (PCMB) in the outer bathing solution (1 mM) on the rate of ^{24}Na discharge from epithelium into the outer bathing solution (J^{Na}_{eff}) in short-circuited skins bathed on both sides by Na_2SO_4-Ringer's solution, with high cell Na concentration induced by long term effect of ouabain. Na isotope was added to the inner compartment at time zero. Amiloride was used at 10^{-4} M concentration. J^{Na*}_{eff} is the steady-state value prior to the addition of PCMB ($n=8$)

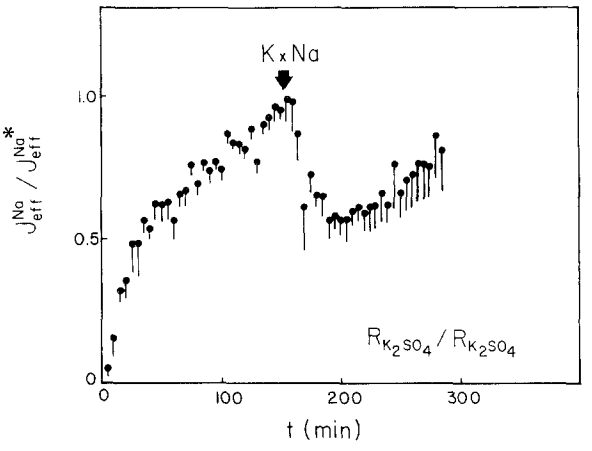

Fig. 6. Effect of K by Na substitution in the outer bathing solution on the rate of ^{24}Na discharge from epithelium into the outer bathing solution (J^{Na}_{eff}) under the condition of very low cell Na concentration and approximate short circuiting of the outer barrier. Skins were bathed on both sides by K_2SO_4-Ringer's solution. Na isotope was added to the inner compartment at time zero. J^{Na}_{eff} is the highest J^{Na}_{eff} value prior to the ionic substitution ($n=7$)

values. Amiloride addition (10^{-4} M in the outer bathing medium) after the effect of PCMB was completed, had a clear effect upon J^{Na}_{eff}, reducing it from 4.35 ± 0.15 nmol cm^{-2} min^{-1} to 1.37 ± 0.39 nmol cm^{-2} min^{-1}, ($P<0.01$, paired t test, $n=6$).

The reaction of the outer skin surface with PCMB again renders the skin amiloride sensitive, as compared to groups with high cell Na concentration, where amiloride had no inhibitory effect upon J^{Na}_{eff} (*see* Fig. 1*a*).

7. Effect of Step Rise in Outer Bathing Solution Na Concentration on J^{Na}_{eff} under the Condition of Very Low Cell Na Concentration and Approximate Short-Circuiting of the Outer Barrier

These experiments were performed with skins equilibrated for at least 1 hr with K_2SO_4-Ringer's solution bathing both sides of skin. The inner solution contained 0.5 mM Na to prevent any unspecific binding of ^{24}Na, when this isotope is added to the inner compartment. High K concentration in the inner compartment was used to depolarize the inner barrier and to increase its electrical conductance, in order to permit an approximate voltage clamping of the outer barrier to be carried out by clamping the whole skin (Morel & Leblanc, 1975; Fuchs et al., 1977).

In the present experimental condition, the absence of Na in the outer medium drastically lowers cell Na concentration (Rick, Dörge, von Arnim & Thurau, 1978) and, as a consequence, increase the Na permeability of the outer barrier by removing the Na self-inhibition, offering, therefore, optimum condition to test the effect of Na ions on the permeability of the outer barrier.

A step rise in outer Na concentration from zero to 115 mM, by equimolar substitution of K, drastically reduces J^{Na}_{eff}, as shown in Fig. 6, which is then followed by a partial recuperation with time.

Discussion

The aim of the present work was to study the rate of ^{24}Na discharge from epithelium into the outer bathing solution (J^{Na}_{eff}) induced by maneuvers which would lead us to a better knowledge of the parameters involved in the regulation of the outer barrier Na permeability (P^{Na}_o). The same kind of approach was successfully applied to the study of ^{42}K discharge across the same barrier (Varanda & Lacaz-Vieira, 1978, 1979; Lacaz-Vieira et al., 1979). Obviously, the epithelium being a double barrier series membrane system, we are aware that the results could, in some circumstances, be influenced by the inner barrier or by changes in tight-junction Na permeability, as will be discussed along with the specific points.

There are controversial opinions in the literature regarding Na efflux in amphibian skin: its pathways

and its behavior in different experimental conditions and for different animal species. Rick, Dörge and Nagel, (1975) reported that the Na efflux in skins of *Rana temporaria* and *Rana esculenta* remained unchanged after amiloride and ouabain and concluded that it follows mainly an extracellular pathway. On the other hand, Biber and Mullen (1976) suggested that the Na efflux in skins of *Rana pipiens* proceeds through a transcellular route that interacts with the active transport pathway. Our results with NaCl-Ringer's solution on both sides of Skin (Section 1) are in agreement with observations of Beauwens, Noé and Crabbé (1978) in the skin of *Bufo marinus* in NaCl-Ringer's solution and indicate that in this experimental condition almost half of J_{eff}^{Na} goes through a transcellular route and the rest via a paracellular pathway.

Experiments reported in Section 2.1 indicate that with NaCl-Ringer's solution bathing both sides of skin, ouabain induces a large but transient increase of J_{eff}^{Na}. An increase of the Na efflux after ouabain treatment has been described in the literature and given different explanations. Biber and Mullen (1977) in skins of *R. pipiens* and Beauwens et al. (1978) in skins of *B. marinus* interpreted it as due to ouabain inhibiting Na isotope recirculation by a pump-leak mechanism present in the basolateral membranes of the epithelial cells. Hviid-Larsen (1972), in skins of *B. bufo*, postulated that ouabain changes the Na/K pump into a Na/Na exchange mechanism. Rick et al. (1975), on the other hand, reported that the Na efflux in skins of *R. temporaria* and *R. esculenta* is ouabain insensitive. The results so far presented indicate that the differences reported in the literature could, in part, be ascribed to differences in the amphibian species used in the experiments.

Our results indicate that the transient increase of J_{eff}^{Na} under the effect of ouabain (Section 2.1) is due to a transient increase of the transcellular component of J_{eff}^{Na}, since amiloride tested at the peak of this increase (Fig. 1*c*) sharply reduces it. The increase of J_{eff}^{Na} induced by ouabain, which is followed by a decline with time, is compatible with at least two processes influencing the transcellular component of J_{eff}^{Na}; one responsible for its increase and the other, with a later onset, inducing a decline with time. The initial increase of J_{eff}^{Na}, after ouabain is added to the system, starts soon after its addition and can be interpreted as due to depolarization of the outer barrier, which would reduce the electrical force against the efflux of any positively charged species crossing the outer barrier. Thus, the behavior of J_{eff}^{Na} is similar to that shown by the efflux of ^{42}K (J_{eff}^{K}) which also increases under the effect of ouabain (Varanda & Lacaz-Vieira, 1979; Lacaz-Vieira et al., 1979). This depolarization would be a consequence of ouabain blocking the electrogenic Na pump that has been demonstrated in this preparation (Lacaz-Vieira et al., 1979, Varanda & Lacaz-Vieira, 1979) and in the skins of *R. temporaria, R. esculenta,* and *B. marinus* (Nagel, 1980). The depolarizing effect of ouabain on the outer barrier, as a consequence of its action at pump level, is due to the electrical coupling of outer and inner barriers by the voltage-clamp condition.

After ouabain, the effect of amiloride upon J_{eff}^{Na} depends on the length of time ouabain was in contact with the skin. Thus, Fig. 1*c* shows that in the J_{eff}^{Na} peak induced by ouabain, amiloride added to the outer medium drastically, and almost immediately, reduces J_{eff}^{Na} to a value approximately equal to the control steady-state value prior to the action of ouabain. On the other hand, in the steady-state condition attained by J_{eff}^{Na} after ouabain, the effect of amiloride is null (Fig. 1*a*) or very small (Fig. 1*b*). The fact that the J_{eff}^{Na} amiloride-insensitive component is about half of the control steady-state value observed with NaCl-Ringer's solution on both sides of skin (Section 1), is almost equal to the control value prior to ouabain, when amiloride is tested in the J_{eff}^{Na} peak induced by ouabain (Fig. 1*c*), and is close to double the control value prior to ouabain, when amiloride is tested after long time exposure to ouabain (Fig. 1*b*), may indicate that the amiloride-insensitive component slowly increases with time after ouabain treatment. We cannot, for the moment, discriminate between an increase in the paracellular component of J_{eff}^{Na} or the appearance of amiloride-insensitive channels in the apical barrier of the most superficial epithelial cells, to interpret the slow increase of the amiloride-insensitive component of J_{eff}^{Na} after ouabain treatment.

To interpret the decline of J_{eff}^{Na} after the peak induced by ouabain, we are postulating a reduction of P_o^{Na} with time (as already shown for the first time by Erlij and Smith (1973) in Na uptake measurements) since pump block would certainly lead to an increase of cell ^{24}Na concentration and to a depolarization of the outer barrier (Nagel, 1980) and these two changes would be expected to increase the rate of ^{24}Na discharge from cell to the outer compartment. However, we cannot rule out the possibility that a reduction of the Na permeability of the basolateral membrane could also contribute to the slow decline of J_{eff}^{Na} with time after the peak induced by ouabain. As the Na concentration in the outer bathing solution was always kept high and constant in the ouabain experiments (Section 2.1), the results strongly suggest that the rise in cell Na concentration induced by ouabain, as shown by Rick et al. (1978) in skins of *R. temporaria* and *R. esculenta,* is the signal that determines closure of outer barrier Na channels. It is also

conceivable that this effect could be mediated by a rise in cell Ca^{++} concentration (Taylor & Windhager, 1979; Taylor, 1980).

Our results, indicating that cell Na concentration might play an important role in regulating P_o^{Na}, as suggested first by Erlij and Smith (1973), are in clear contrast with the interpretation of Fuchs et al. (1977) and of Van Driessche and Lindemann (1979) which considers the external Na concentration, and not the cellular Na concentration, the variable that controls the outer barrier Na permeability.

The effect of PCMB added only to the outer bathing solution (Section 6, Fig. 5) in a condition where amiloride is without effect, increasing J_{eff}^{Na} and making it now amiloride sensitive, clearly shows that the reduction of J_{eff}^{Na} after the peak induced by ouabain is due to closing of the apical membrane Na channels and can be taken as a strong argument against the alternative interpretation, that the J_{eff}^{Na} decline which follows the peak induced by ouabain is due to a decline of the Na permeability of the basolateral membrane, since it would be improbable that the large sulphydryl reagent molecule, added to the outer compartment, would cross the outer barrier without affecting it, to react with the basolateral membrane.

It could be argued that the absence of amiloride effect, as shown in Fig. 1*a*, could reflect a reduction of binding characteristics at the amiloride binding sites, due to a rise of Na concentration in contact with the cytoplasmic face of the apical membrane, as proposed by Shum and Fanelli (1978). This, however, would not by itself explain the decline of J_{eff}^{Na} after the peak induced by ouabain.

The experiments of Section 2.2 clearly show the importance of the presence of Na in the outer bathing solution on the transient increase of J_{eff}^{Na} induced by ouabain (Section 2.1) since Na-free outer bathing solution (K replacing Na) completely abolishes the effect of ouabain upon J_{eff}^{Na}. The absence of Na being transported by the active pathway, due to its substitution by K in the outer bathing solution, would be expected to create a condition of maximal Na recirculation across the inner barrier. This recirculation, however, seems not to be significant, as we do not observe any effect of ouabain upon J_{eff}^{Na} with Na-free outer bathing solution. These results strongly support our hypothesis that the increase of J_{eff}^{Na}, due to ouabain action in experiments with Na in the outer bathing medium, is mediated by a depolarization of the outer barrier. This increase would not be expected to occur in the absence of Na being transported across the skin, since the electrogenic Na pump, as already discussed, is inoperative and its inhibition by ouabain would not be expected to induce any additional effect.

Finally, it is interesting to notice that J_{eff}^{Na} observed with Na-free outer bathing medium is significantly higher than that with Na in the outer bathing medium. In the control condition, with Na in the outer bathing medium, the steady-state J_{eff}^{Na} is 1.07 ± 0.22 nmol cm^{-2} min^{-1} ($n=18$) (Section 1). With Na-free solution bathing the outer skin surface for at least 2 hr before addition of Na isotope, the steady state reached by J_{eff}^{Na} is much higher (8.70 ± 3.1 nmol cm^{-2} min^{-1}, $n=7$) than in the control, with Na in the external medium. This large difference in J_{eff}^{Na} is consistent with the interpretation that in the absence of external Na, the cell Na concentration is very low, almost zero (Rick et al. 1978) and, as a consequence, the permeability of the outer barrier – controlled by cell Na concentration – is at its maximal value. However, when amiloride is tested in both groups mentioned above, it can be seen that the amiloride-insensitive component of J_{eff}^{Na} is also elevated in the group with Na-free external bathing solution (Fig. 2). This large amiloride-insensitive component could be a consequence of a higher paracellular component or the appearance of amiloride-insensitive pathways across the apical border of the superficial cell layer. We have no reasons to assume that the elevation of the transcellular component with Na-free external solution could be a consequence of an increase of Na movement across the inner barrier, from inner solution to cell compartment, since, according to electron microprobe data of Rick et al. (1978) in *R. esculenta* end *R. temporaria*, in the presence of Na-free external solution, cell Na concentration is reduced to almost zero in all cell layers.

The effects of long-term exposure of the outer skin surface to Na-free solution upon J_{eff}^{Na} should be compared to the effect of a step change from a Na to a Na-free external solution (Section 3). Na removal from the outer compartment causes a step decline in J_{eff}^{Na} from 1.17 ± 0.10 nmol cm^{-2} min^{-1} to 0.90 ± 0.06 nmol cm^{-2} min^{-1} ($P=0.01$, paired t test, $n=20$). We consider that this decline of J_{eff}^{Na} is a consequence of a hyperpolarization of the outer barrier, as already discussed. A few experiments indicate that if a long time elapses after K replaces Na in the outer solution, then J_{eff}^{Na} displays a tendency to increase, which is consistent with the results presented in Section 2.2

When a step change from a Na to a Na-free external solution is performed in the peak of J_{eff}^{Na} induced by ouabain (Section 4), an increase of J_{eff}^{Na} is observed, instead of a decline, as described in Section 3. These results are expected and consistent with the electrophysiological data of Helman and Fisher (1977) in frog skin and of Nagel (1980) in frog and toad skins showing that 3 to 5 min after addition of ouabain

to the inner solution, the hyperpolarization of the outer barrier which follows external Na by Na-free solution substitution in the outer medium in control conditions is no longer seen. Consequently, the increase of J_{eff}^{Na} is consistent with an increase of P_o^{Na} due to a decline in cell Na concentration, in the vicinity of the cytoplasmic surface of the apical membrane of the superficial cells of the epithelium, which should follow Na by Na-free solution substitution in the outer medium. This reduction of cell Na concentration, in a region close to the apical membrane, would be expected to start within seconds after the ionic substitution and would be due to leakage of Na from cell compartment to the Na-free outer solution, according to results of Morel and Leblanc (1975) in *R. esculenta.*

In order to be able to see changes in P_o^{Na} induced by changes in Na concentration unperturbed by simultaneous changes in the electrical potential difference across the outer barrier, experiments were performed in a voltage-clamp condition of the outer barrier, carried out by clamping the whole skin (Morel & Leblanc, 1975; Fuchs et al., 1977) (Results, Section 7). A step rise in the outer solution Na concentration, from Na-free solution to Na 115 mM, drastically reduces J_{eff}^{Na} as shown in Fig. 6, which is then followed by a partial recuperation with time. This late recuperation could well be a consequence of ^{24}Na build up into the cell compartment due to reduction of its rate of discharge across the outer barrier. On the grounds of previous discussion, we consider that in this case also the inhibitory effect of raising outer solution Na concentration is due to a consequent increase of Na concentration in the cell compartment, in a region adjacent to the apical membrane.

The absence of amiloride effect upon J_{eff}^{Na} in a condition of high cell Na concentration and voltage clamp of the outer barrier (Section 5, Fig. 4*a*) is in complete agreement with a potent and complete block of outer barrier Na channels induced by high cell Na concentration. The amiloride-insensitive component of J_{eff}^{Na}, by reasons which are now being investigated, is much higher than in the control situation with Na present in the inner bathing solution.

The results with PCMP (Section 6, Fig. 5) contribute as a strong argument in favor of the interpretation that the Na channels of the outer barrier are completely blocked by high cell Na concentration, since PCMB at 1 mM concentration in the outer solution, acting at the outward-facing side of the outer barrier, removes the Na channel inhibition by high cell Na concentration and makes the Na channels again amiloride sensitive. The results of the action of PCMB upon J_{eff}^{Na} are in consonance with the electrophysiological observations of Dick and Lindemann (1975) and of Fuchs et al. (1977) in frog skin, except for the fact that those authors assume that Na inhibition upon its own channels is due to an action on the outward-facing surface of the apical membrane, as proposed also by Van Driessche and Lindemann (1979). With Na-free outer bathing solution, a condition where the cell Na concentration is almost zero (Rick et al., 1978), addition of PCMB to the outer compartment is completely without effect upon J_{eff}^{Na} (S. Sanioto and F. Lacaz-Vieira[1]), indicating that the increase of J_{eff}^{Na} by PCMB shown in section 6, Fig. 5, is due to a release of Na self inhibition upon its channels in the outer barrier.

In some skins, with the Na channels completely blocked by high cell Na concentration, amiloride showed an effect opposite to that which would be expected from its known inhibitory effect on the Na conductance of the outer barrier (Bentley, 1968; Ehrlich & Crabbé, 1968; Dörge & Nagel, 1970; Nagel & Dörge, 1970; Salako & Smith, 1970*a*, 1970*b*; Biber, 1971; Cuthbert, 1973; Moreno et al., 1973; Cereijido, Rabito, Rodriguez Boulan & Rotunno, 1974; Cuthbert & Shum, 1974*a*, 1974*b*, 1975, 1976, 1977; Crabbé, 1980), increasing J_{eff}^{Na} (Section 5, Fig. 4*b*). It is conceivable that the amiloride molecule could have a moiety that stimulates Na transport across the outer barrier, which is, however, much less powerful than its inhibitory portion. Due to the particular condition of this experimental group, with the Na channels already blocked by high cell Na concentration, the stimulatory effect could now be seen. The stimulatory effect upon J_{eff}^{Na}, somehow, resembles the stimulatory effect upon short-circuit current of the amiloride analogue dimethylated at position 5 (Li & DeSousa, 1979). Also, in the gills of *Anguilla anguilla*, Cuthbert and Maetz (1972) showed that amiloride itself enhances Na permeability. We could not, however, rule out the possible effect of amiloride opening tight junctions to explain the results shown in Fig. 4*b*. However, if amiloride had an effect upon tight junctions, it would be expected to be in the opposite direction, according to results of Balaban, Mandel and Benos (1979) in gallbladder epithelium. Amiloride effect upon the inner barrier, leading to an increase of J_{eff}^{Na} seems unprobable since inner and outer barriers are electrically uncoupled by the voltage clamp of the outer barrier (Morel & Leblanc, 1975; Fuchs et al., (1977). In addition, the fact that J_{eff}^{Na} starts to increase almost immediately after amiloride is added to the outer bathing solution is also a strong argument against any thinkable effect of amiloride increasing Na permeability of the inner barrier, since amiloride

[1] *Unpublished observation*

first had to enter the cell, affect P_i^{Na}, lead to an increase of cell ^{24}Na concentration, to finally cause an increase of J_{eff}^{Na}.

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