Biochimica et Biophysica Acta, 311 (1973) 630–639

BBA 76382

EFFECT OF THE COMPOSITION OF THE INNER BATHING SOLUTION ON TRANSPORT PROPERTIES OF THE FROG SKIN

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(Received December 4th, 1972)

(Revised March 20th, 1973)

SUMMARY

1. Electrical parameters and unidirectional Na^+ fluxes were measured in isolated frog skin bathed by Ringer's solutions of different composition. Since the attention was focused on changes elicited by modifications of the inner bathing Ringer, the composition of the outer solution was usually kept constant, *i.e.* isotonic Ringer with 50 mM NaCl.

2. The reduction of the concentration of NaCl on the inside from 115 mM to 10 mM (keeping the osmolarity constant) produces a marked decrease of the short circuit current (SCC), electrical potential difference ($\Delta\psi$) and electrical conductance ($\varkappa$), with an inflection point around 50 mM. This position of the inflection point is not appreciably shifted by using Ringers with 30, 50 or 80 mM NaCl on the outside, or by replacing Cl^- with SO_4^{2-}, or by making the Ringers hypertonic.

3. The decrease of SCC caused by the reduction of NaCl from plasma levels (115 mM) is due to reductions of the Na^+ influx and of the Cl^- efflux.

4. Although the decrease of the concentration of Na^+ and of Cl^-, and the increase of the effective osmolarity of the inner bathing solution, can elicit a drop of SCC individually, the maximal decrease of SCC is obtained when the three factors act together, *i.e.* when NaCl is replaced by an equivalent amount of sucrose.

5. Under physiological conditions the concentration of NaCl of the inner bathing solution is close to 115 mM, and the concentration of the outer solution is much lower. In experiments *in vitro* it is general practice to reduce the concentration of the inner bathing solution in order to study ion penetration in the absence of concentration gradients. The present results indicate that under these circumstances part of the observed kinetics may be due to effects elicited by changes of the inner solution.

INTRODUCTION

In order to study the mechanism that carries out the transport of ions across epithelia, it is customary to mount the frog skin between two identical bathing

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Abbreviation: SCC, short circuit current.

solutions as this greatly simplifies the techniques, and the interpretation of the observed phenomena. Since the frog skin can transport Na^+ from an outer solution with low concentration of Na^+, the study of the mechanism with identical solutions requires the use of a low concentration of NaCl on the inside too. In this case the osmolarity of the solutions is kept constant by adding choline chloride, sucrose, Na_2SO_4, *etc.*, as it was shown that changes in the osmolarity of the Ringer and the cellular volume bring about modifications of the electrical parameters of the frog skin[1,2]. In the course of studies of Na^+ transport at low concentration of Na^+ on the outside sometimes we wanted to take advantage of the technique developed by Ussing and Zerahn[3] and have used low concentrations of NaCl on the inside keeping the osmolarity constant, and at other times we wanted to study Na^+ transport under conditions resembling the natural situation of the skin, and have used Ringer with 115 mM NaCl on the inside. We have observed that there is a marked difference between the two conditions, indicating that the whole mechanism is very sensitive to changes of the concentration of NaCl on the inside. In order to explore this in a more systematic manner we have performed the series of studies reported in the present paper. It consists essentially of the study of the influence of the nature of inner solution on the short circuit current, voltage, electric conductance and unidirectional fluxes. It is shown that these parameters are very sensitive to the osmolarity, concentration of Na^+, and concentration of Cl^- of the inner solution taken either individually or in combination. The curves relating these parameters to the concentration of NaCl on the inside exhibit a sharp inflection around 50 mM NaCl.

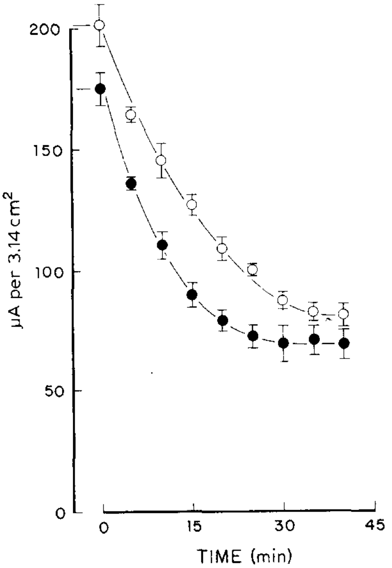
MATERIAL AND METHODS

The abdominal skin of the South American frog *Leptodactylus ocellatus* L. was used. The skin was mounted as a flat sheet in a lucite chamber as described in detail elsewhere[4,5]. The electrical potential difference was measured with a Keithley 200 B electrometer, and the short circuit current with the technique described by Ussing and Zerahn[3].

Unidirectional Na^+ fluxes were measured with $^{22}Na^+$ and $^{24}Na^+$ as before[4,5]. Countings were made in a Nuclear Chicago Auto Gamma well-type scintillation counter set as spectrometer at the corresponding sodium peak.

The control Ringer solution used contained (mM): 115 NaCl, 2.4 $KHCO_3$, 1.0 $CaCl_2$, 1.0 glucose. It was continuously gassed and stirred with moist air. The pH was 8.2, the temperature of the room was conditioned to 20 °C. Other solutions used are described in Results.

The basic experimental protocol was to mount the skin with Ringer using 115 mM NaCl on the inside, and 50 mM NaCl on the outside, and allow an equilibration period until the electrical parameters, recorded every 5 min, achieved a steady value. This concentration was adopted so as to be able to test later higher and lower concentrations within the range of isotonic Ringers. Sucrose was added to the outer solution so it had the same osmolarity as the inner one. The values recorded under this condition were taken as control. Every solution tested was interpolated between two measurements under control conditions. Fig. 1 illustrates the time course of the short circuit current following a change in the concentration



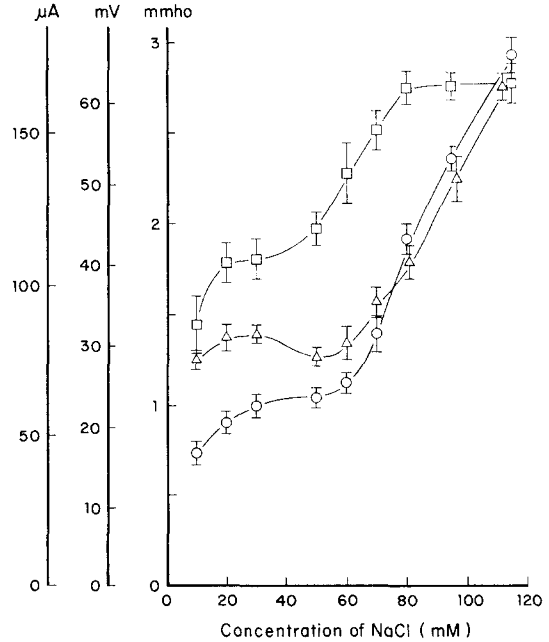


Fig. 1. Time course of the change in short circuit current following a change, at zero time, of the concentration of NaCl on the inside from 115–50 mM. The Ringer solution on the outside has 50mM Na^+. The main anion on the outside is SO_4^{2-} (○) and Cl^- (●). The osmolarity of the Ringers is kept constant at 230 mosM by adding sucrose. All experimental values in this and following figures represent mean ± standard errors of 8–10 determinations.

Fig. 2. Electrical potential difference (□), conductance (△) and SCC (○) as functions of the concentration of NaCl on the inside. The outer Ringer has 50 mM NaCl. The osmolarity is kept constant by adding sucrose.

of NaCl in the inner solution from 115–50 mM. There are two series of experiments in Fig. 1: one carried out with 50 mM NaCl Ringers on the outside (full circles) and another carried out with 25 mM Na_2SO_4 Ringers on the outside (open circles). All Ringers were isotonic (230 mosM). Changes are essentially completed in less than 30 min. In a given set of experiments each skin was used to test the whole series of concentrations.

The following nomenclature is used: short circuit current (SCC), electrical potential difference ($\Delta\psi$), and electrical conductance ($\varkappa$). Subscript *o* referes to the outer, and subscript *i* to the inner bathing solutions. Results are expressed as mean ± standard error (number of observations).

RESULTS

Fig. 2 illustrates the SCC, $\Delta\psi$ and $\varkappa$ as functions of the concentration of NaCl in the inner bathing solution: $NaCl_i$. The osmolarity is kept constant with sucrose. As the concentration of $NaCl_i$ is lowered there is a marked decrease of SCC, $\Delta\psi$ and $\varkappa$. Since the concentration of NaCl on the outside is kept constant at 50 mM, in those points where the concentration of the inner bathing solution is above 50 mM part of the current may be due to the short circuiting of a Cl^- diffusion potential. In this respect it suggests that the curve of SCC shows a sharp inflection point around 50 mM. This point was investigated in a series of studies carried out with 30 mM NaCl on the outside (Fig. 3, full circles) and also with 80 mM (Fig. 3, open circles). The curve in Fig. 3 is just a copy of the SCC curve in Fig. 2 and was drawn to facilitate the comparison. It may be observed that the points of 30 and of 80 mM of $NaCl_0$ do not depart markedly from the curve obtained at 50 mM and that there is no apparent shift in the inflection point. While this does not discount that part of the drop in SCC may be due to a decrease of an outward diffusion potential of Cl^-, it makes it unlikely that the inflection point would correspond to the point where the concentration gradient across the skin is eliminated. MacRobbie and Ussing[1] and Ussing[6] have found that replacement of NaCl in the inner bathing solution for a less permeable solute produces shrinkage of the epithelium accompanied by a decrease of SCC. Therefore the effect observed in the SCC may be due in part to a shrinkage elicited by the replacement of NaCl by sucrose. Similar effects elicited by the nature and concentration of the anion on the inside were found by Huf[7] in frog skin and by Steinmetz *et al.*[8], Gonzalez *et al.*[9] and Singer *et al.*[10] in the urinary bladder. Ussing and Windhager[11] demonstrated that if the Ringer solutions are made hypertonic, an increase in the leak between the

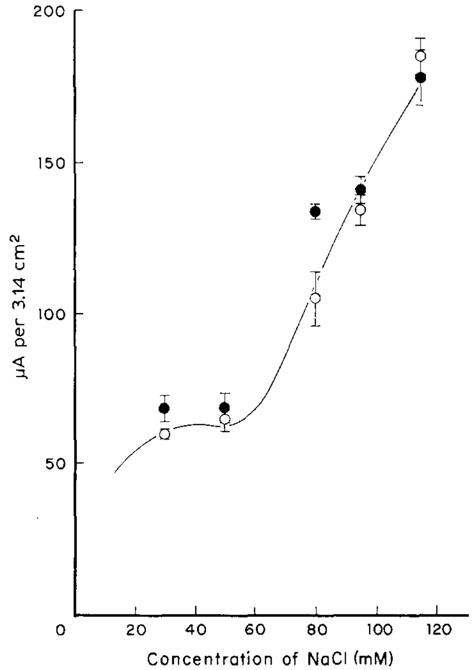


Fig. 3. SCC as a function of the concentration of NaCl of the inner bathing Ringer. The outer solution has 30 mM NaCl (●) or 80 mM NaCl (○). The osmolarity of the Ringers are kept constant by adding sucrose. The line is a reproduction of the SCC curve of Fig. 2 and was now drawn to fit the experimental points of Fig. 3, but to permit comparisons with data obtained with 50 mM NaCl on the outside.

cells occurs which causes a further increase in κ. This is confirmed in Figs 4a, 4b and 4c which illustrate the same kind of experiment as the ones described above, except that this time the Ringers were made hypertonic with sucrose on both sides (300 instead of 230 mosM) so that there was no osmotic gradient across the skin. For the sake of comparison in all these figures the control curves are copies of the corresponding ones in Fig. 2. It may be observed that the shape of the SCC, κ and $\Delta\psi$ curves with hypertonic Ringers is the same as with isotonic solutions. This indicates that the changes elicited by lowering $NaCl_i$ are not basically due to changes in osmolarity.

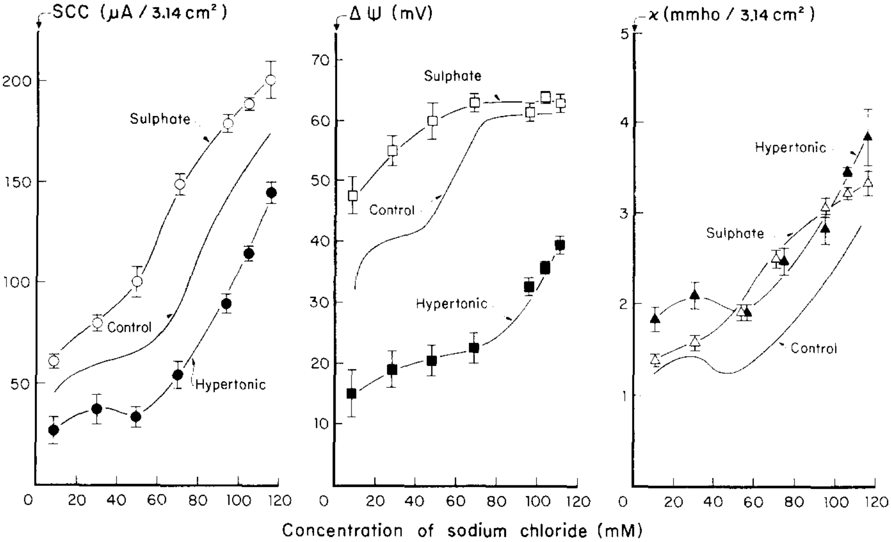
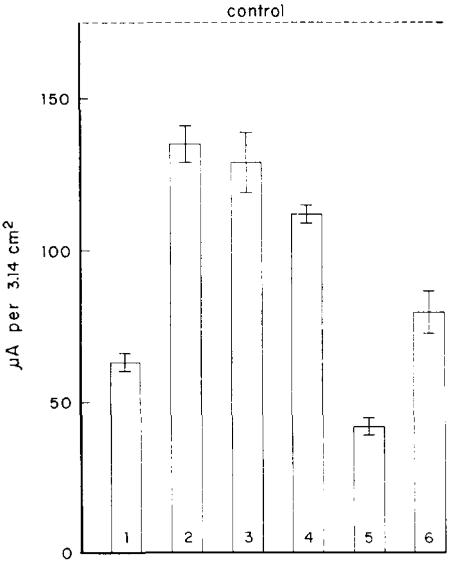


Fig. 4. (a) Short circuit current (○); (b) voltage (□); and (c) conductance (△) as functions of the concentration of NaCl on the inside. The bold curve in each case reproduces the corresponding curve of Fig. 2. Curves labelled sulphate correspond to experiments in which the outer Ringer is isotonic and contains 25 mM Na_2SO_4. Curves labelled hypertonic corresponds to experiments with chloride Ringers in which the osmolarity was increased on both sides from 230–300 mosM by adding sucrose.

The nature of the anion bathing the outer face does not seem to bear a fundamental role on the nature of the phenomenon elicited by changes in the inner solution. Fig. 4 (open symbols) illustrates a series of experiments in which the outer solution was a sulphate Ringer. The skin of *L. ocellatus* has an inward transport of Cl^- (ref. 12) which makes the SCC smaller than the net Na^+ transport. Therefore the replacement of Cl^- by SO_4^{2-} on the outside results in an increase in the SCC (Fig. 4a), but the curve of SCC as a function of $NaCl_i$ follows the same general pattern with SO_4^{2-} as with relative minimum exhibited by the control curve at 50 mM (Figs 4b and 4c).

The experiments described so far suggest that, even when the nature of the outer solution may quantitatively affect the parameters measured, the shape of the SCC, κ and $\Delta\psi$ curves is primarily related to situations on the inside of the skin. In an attempt to gain some insight on this influence a series of studies were made in which the outer solution was an isotonic (sucrose) Ringer with 50 mM NaCl, and the inner solution was changed for the ones listed in Fig. 5. The control line refers to the value of the SCC obtained with 115 mM NaCl Ringer on the inside. Column 1 shows the inhibition observed when the $NaCl_i$ is reduced from 115–50 mM and replaced with sucrose, and which was already discussed in Fig. 2. Column 2 shows that, if the concentration of chloride is kept constant the decrease in SCC is much smaller but there is still a definite inhibition. Thus the decrease of the concentration of Na^+ on the inside seems to be in part responsible of the decrease in SCC.



	Sucr	Chol	Sucr	–	Sucr	–
Sodium	50	50	115	50	50	50
Chloride	50	115	50	50	0	0
Sulphate	–	–	32.5	–	25	25
Osmolarity	ISO	ISO	ISO	HIPO	ISO	HIPO

Fig. 5. SCC as function of the composition of the inner solution. In all cases the outer Ringer contains 50 mM NaCl, and is maintained isotonic by adding sucrose. The control line corresponds to the SCC measured under control condition, *i.e.* with 115 mM NaCl on the inside. The columns represent the values obtained with the different Ringers listed underneath. Besides of the component listed, all Ringers' solutions contained 2.4 mM $KHCO_3$ and 1.0 mM $CaCl_2$.

Column 3 shows the converse situation: Na^+ is kept constant, but Cl^- is reduced. The reduction of Cl^- seems to be equally effective in inhibiting the SCC as is the reduction of Na_i. As discussed above, the change of NaCl by a less permeable solute, leads to a shrinkage of the epithelial cells and this shrinkage, elicited by sucrose, is regarded as the key feature in the decrease of SCC. Yet Column 4 shows that even if the removal of NaCl is not compensated with sucrose and the Ringer is made hypotonic (*i.e.* no sucrose-induced shrinkage expected), there is a significant decrease of SCC. Thus the decreaes of ${Na^+}_i$ does not imply that the tonicity has no effect. On the contrary, by comparing Columns 5 and 6 it is evident that if the removal of Na^+ and Cl^- is made under hypotonic condition (Column 6),the stimulation produced by the hypotonicity (Column 6) tends to compensate the severe inhibition brought about by the removal of the ions (compare also Columns 1 and 4).

The position of the inflection point observed around 50 mM in almost all curves (in some of the curves it is a relative minimum) was not appreciably shifted by changing NaCl (Fig. 3), nor by changing the anion, nor by modifying the tonicity of the solutions (Fig. 4). Fischbarg *et al.*[13] have observed an inflection point at 60 mM in the curve relating Cl^- influx to Cl^- concentration on both sides.

Flux measurements

The aim of the experiments in this section is to study the influence of the change in $NaCl_i$ on the unidirectional fluxes of Na^+ as determined simultaneously with $^{22}Na^+$ (influx) and $^{24}Na^+$ (efflux) (Fig. 6). The value of the influx (1.91 ± 0.09 (8) $\mu mole \cdot h^{-1} \cdot cm^{-2}$ and of the efflux (0.173 ± 0.035 (8) $\mu mole \cdot h^{-1} \cdot cm^{-2}$) are close to the values obtained previously in skins of *L. ocellatus*[12,14,15]. The reduction of $NaCl_i$ from 115–50 mM decreases the influx as well as the efflux of Na^+. The net flux is also reduced from 1.73 ± 0.09 (8)–1.18 ± 0.11 (8) $\mu mole \cdot h^{-1} \cdot cm^{-2}$. With 115 mM $NaCl_i$ the SCC is larger than the net Na^+ flux (3.16 *vs* 1.73 $\mu mole \cdot h^{-1} \cdot cm^{-2}$). This may be attributed to an excess current that should be passed to cancel the diffusion potential of chloride from the inside (115 mM) to the outside (50 mM). The change of $NaCl_i$ from 115 to 50 mM inhibited the net flux of Na^+ (as determined by the tracer method) and reduced also the excess of SCC over the net Na^+ flux. It is illustrative to reword this conclusion and say that the level of $NaCl_i$ stimulates Na^+ transport across the epithelium. Curran and Cereijido[16] have shown that $NaCl_i$ stimulates also the flux of K^+ from the inner bathing solution to the cells.

DISCUSSION

Several factors might, in principle produce the drop in SCC when $NaCl_i$ is lowered: (a) Decrease in Na^+ influx: this factor is involved as Fig. 6 shows that there is actually a decrease in Na^+ influx. (b) Increase of Na^+ efflux: the data in Fig. 6 show that this factor may be discarded. Na^+ efflux decreases, and this may be easily understood on the basis that the Na^+ efflux is a passive process and, by changing $NaCl_i$ from 115 to 50 mM, the driving force for an outward unidirectional flux is reduced. In connection with this, it may be noticed that the conductance also decreases considerably (Fig. 2). (c) Increase of Cl^- influx: this factor may be discarded as the drop in SCC with 50 mM Cl^- on the outside is 112 μA, and with 25 mM SO_4^{2-} is 100 μA, *i.e.* no marked difference is observed. (d) Decrease in Cl^- efflux: two lines of evidence support a decrease of Cl^- efflux as one of the components of the fall of SCC: (1) Keeping Cl^-_i at 115 mM and only decreasing the Na^+ concentration (Fig. 5, Column 3) elicits an inhibition of the SCC of only 25% (compare with Column 1). (2) When $NaCl_i$ is changed from 115 to 50 mM the discrepancy between net flux and SCC decreases (Fig. 6). In summary this suggests that the ionic movement involved in the inhibition of SCC is mainly a decrease of Na^+ influx and in Cl^- efflux. These ion movements refer to transepithelial fluxes. Diffusion potentials might also arise from the diffusion of ions from the inner (or outer) solution to the cells and *vice versa*. These changes, though, would appear as

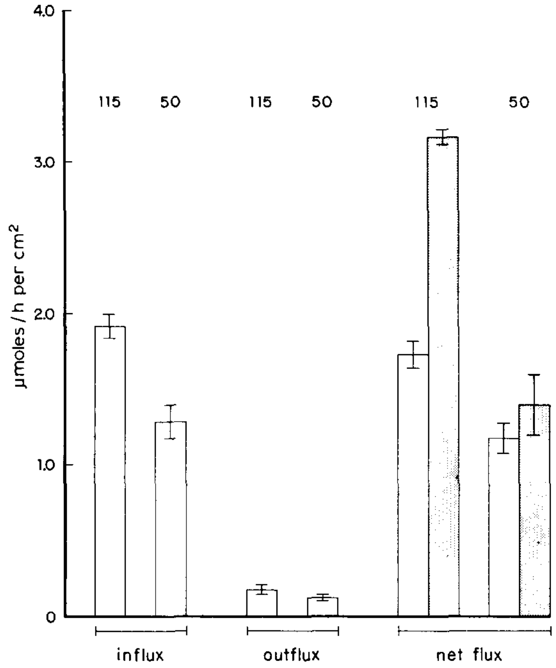


Fig. 6. Unidirectional fluxes of Na$^+$ as measured with ^{24}Na$^+$ and ^{22}Na$^+$. The shaded columns correspond to the conversion of SCC to µmole·h^{-1}·cm^{-2}. Each determination was made with Ringer containing 115 and 50 mM NaCl on the inside. The outer solution contains always 50 mM NaCl. All Ringer solutions have the same osmolarity (230 mosM).

transients and would not be established with the long delay which is characteristic of the phenomenon under consideration (see Fig. 1). This point is further discussed below.

The direct effect of anions on Na$^+$ transport was clearly demonstrated in frog skin and cornea by Zadunaisky and coworkers[12,13,17] and by Tonnies Gil Ferreira[2]. This author has also shown that the ionic dependence of Na$^+$ transport is not exclusive for Cl$^-$. Smith *et al.*[18] carried out a detailed study of this anion selectivity. Conversely, Cl$^-$ permeability depends on ambient concentration of NaCl[19]. Zadunaisky and de Fisch[17] suggested that Na$^+$ and the associated anion are both transported in certain regions of the epithelium, the anion being restricted and not showing a complete transepithelial transport. These observations suggest that the Na$^+$ and Cl$^-$ causing and experiencing the influence of the changes in NaCl concentration are those coming from the outside. Yet the effect we have been describing in this paper is elicited from the inside, suggesting that there must be another mechanism involved besides of those just discussed.

Changes in the composition of the inner bathing solution are known to influence the transport of Na$^+$ across. Thus hypotonic solutions bathing the inside, cause swelling of the cells and an increase in SCC[1,6]. On the other hand shrinkage leads to inhibition of SCC[6]. Shrinkage alone cannot explain the inhibition of the SCC that follows a reduction of NaCl as it seen (see Fig. 5, Column 4) that the removal of NaCl leaving the Ringer hypotonic elicits also an inhibition of the SCC.

However the tonicity of the inner bathing solution does vary the SCC in the manner predicted by MacRobbie and Ussing[1] (Fig. 5, compare Column 1 with 4, and 5 with 6).

The long delays observed in Fig. 1 may not be due to a delay in diffusion in the connective or in the intercellular space, but must reflect the time course of a cellular modification. The observation that the transient is longer than the time required to equilibrate the composition of the intercellular space with the solution present on the inside, suggests also that the changes in SCC, $\varkappa$, and $\Delta\psi$ observed may not be ascribed to diffusion potentials at the levels of the tight junctions which close the outermost end of this space. This suggestion is stressed by the fact that changing $NaCl_0$ from 30 to 80 mM does not modify the observed phenomena (Fig. 3).

Besides characterizing the influence that changes in the inner solution have on ion movement, the results may also give the impression of referring to an artifact produced by mounting the skin with different Ringers on each side. In this respect one has to keep in mind that in fact, the common practice of mounting the skin between two identical Ringers with 115 mM NaCl, in spite of constituting one of the most fruitful techniques in membrane research[3] is not the only condition of interest to the membrane biologist. Rather, the condition which would resemble the physiological state more closely is one with 115 mM $NaCl_i$ but with a low $NaCl_0$. The present observation may also bear some influence on the interpretation of observed kinetics. Thus when one desires to study the kinetics of penetration of ions from the outer environment, and in doing so needs to vary the composition of the outer solution, one generally uses the same solution on the inner side too so as to avoid concentration gradients, diffusion potentials, and to take advantage of the technique developed by Ussing and Zerahn[3]. Even when the results reported in this paper emphasize the fact that the behaviour observed under those conditions might not always be attributed to events elicited by the composition of the outer solution, but might be also due to changes on the inner side.

Even though the phenomena described here might be peculiar to *L. ocellatus*, it seems necessary to investigate their possible influence on transport characteristics of other species.

ACKNOWLEDGMENT

We wish to acknowledge our sincere thanks to Dr C. A. Rotunno for helpful discussion, and to Mrs Marisa B. de Gonzalez Avila and Graciela A. de Carman for their valuable assistance. Marcelino Cereijido is a Career Investigator from the National Research Council of Argentina (C.N.I.C.T.). This work was supported by research grants from the C.N.I.C.T. and the Public Health Service of U.S.A.

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