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Influence of Lithium upon the Intracellular Potential of Frog Skin Epithelium

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Summary. The effect of Li upon the intracellular potential of frog skin (*Rana esculenta*) was investigated. In the range between 1 and 25 mM Li in the epithelial bathing solution, a semilogarithmic linear relationship between [Li] and intracellular potential under short circuit conditions was obtained. The intracellular potential at all [Li] is quantitatively sufficient to explain the previously reported accumulation of Li in the intracellular space of the frog skin epithelium (Leblanc, G. 1972. *Pfluegers Arch.* **337**:1) on the basis of a passive entrance step at the outer border. A reduction of the intracellular potential by Li is also observed in the presence of 6 mM Na in the epithelial bathing solution. Consequences regarding the mechanism of uptake of Na across the outer border of the frog skin are discussed.

Key words: Frog skin, intracellular potential, lithium, sodium transport, amiloride.

The movement of lithium (Li) across the isolated frog skin resembles in many aspects the transepithelial movement of Na. Thus, Li was used repeatedly as a tool to investigate the mechanism of transepithelial Na transport (Zerahn, 1955; Hansen & Zerahn, 1964; Biber & Curran, 1970; Leblanc, 1972; Candia & Chiarandini, 1973; Morel & Leblanc, 1975; Reinach, Candia & Siegel, 1976). In one of these studies (Leblanc, 1972) it was concluded from the accumulation of Li in the epithelial cell water that an active uptake step at the outer border of the epithelial cells must exist, since the intracellular [Li] ($[Li]_c$) exceeded the [Li] of the epithelial bathing solution ($[Li]_o$) considerably. The observed values for $[Li]_c$, which were up to ten times that of $[Li]_o$, would require intracellular potentials up to -60 mV for passive distribution. Previous microelectrode measurements, however, demonstrated intracellular potentials of about -15 mV only under short circuit conditions (Biber & Curran, 1970).

Recent reinvestigations of the potential profile of frog skin epithelium (Nagel, 1975, 1976*b*; Helman & Fisher, 1976, 1977) revealed that previously reported microelectrode data were recorded in general from injured cells and did not represent the normal situation. The true intracellular potential of the frog skin is up to -120 mV under short circuit conditions, if Na-free solution incubates the epithelial surface. This observation pointed to the possibility that considerably negative intracellular potentials might exist also at low $[Li]_o$, which, then, would allow passive accumulation of Li in the epithelial cells as originally suggested by Hansen and Zerahn (1964). Furthermore, it can be expected that a comparison of the response of the intracellular potential upon Li and Na might, in view of the similarities between these two ions, elucidate the mechanism of Na entry across the outer border of the frog skin.

Materials and Methods

Abdominal skins of *Rana esculenta* were mounted in a chamber and punctured as previously described in detail (Nagel, 1976*b*). In brief, the skin which was supported by a grid on the corial side, was impaled from the epithelial side perpendicular to the surface by the use of a stepping motor drive manipulator (Narishige Scientific Instr.). The microelectrodes which were prepared from Omega dot® capillaries of 1.5 mm o.d. (Frederick Haer & Co., Ann Arbor) and filled with 1.5 M KCl, had input resistances of 20–40 MΩ and tip potentials of <5 mV. Intracellular location of the microelectrode was identified according to the criteria previously described (Nagel, 1976*b*). In the present investigation, the skins were short circuited throughout. This provided the continuous recording of V_{sc}, the intracellular potential under short circuit conditions, and I_{sc}, the short circuit current. For measurement of the resistances of the different transepithelial pathways, which is necessary for the identification of intracellular location of the microelectrode, the method of Helman and Fisher (1977) was applied.

Ringer's solution, with 110 mM NaCl, 2.5 mM $KHCO_3$, 1 mM $CaCl_2$, 5 mM glucose, pH=8.2, was used to perfuse the corial side. The epithelial side was either rinsed with the same solution, with a solution containing 6 mM Na, or with Na-free solution. Choline was substituted for Na. Li was added to the epithelial bathing solution in concentrations of 1, 2.5, 5, 10 and 25 mM replacing choline. The perfusion rate of ~20 ml/min allowed complete exchange of the epithelial bathing solution within 10 sec.

Mean values are given ±SEM.

Results

Fig. 1 shows the response of V_{sc} and the corresponding changes of the I_{sc} upon increasing the $[Li]_o$. The epithelial side was already incubated in Na-free choline Ringer's for 30 min before the puncture and again in the periods between exposure to Li. The impaled cell is most unlikely

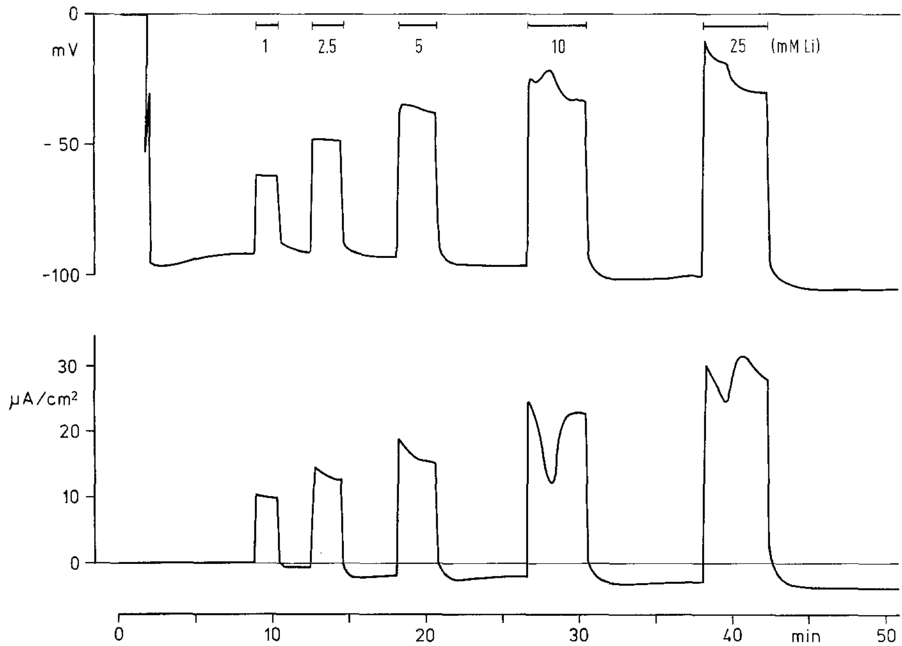

Fig. 1. Record of the intracellular potential of the short circuited frog skin (upper panel) and the short circuit current (lower panel). The epithelial surface was incubated with Na-free choline Ringer's solution. During the periods indicated by bars, Li was applied from the epithelial side substituting for equimolar parts of the choline (redrawn from the original record)

to be located in the first cell layer, since an unstable negative potential, typical for unsuccessful puncture of a cell, was recorded before the accepted stable potential of −91 mV was obtained after further advancement of the microelectrode. Addition of Li to the epithelial bathing solution depolarised V_{sc} in proportion to the $[Li]_o$. Li exerted its effect upon V_{sc} and I_{sc} very fast. More than 90% of the maximal change occurred within the first 5 sec after Li. Omission of Li from the epithelial bathing solution resulted in complete and fast recline of V_{sc} and I_{sc} to control values.

During the choline incubation periods after application of $[Li]_o$ >5 mM, a certain hyperpolarization and a reversed I_{sc} were observed. Hyperpolarization and reversal of I_{sc} increased with increasing $[Li]_o$ and extension of the Li incubation period. Application of amiloride (10^{-4} M) immediately decreased V_{sc} and abolished the reversed I_{sc}, as demonstrated for a typical experiment in Fig. 2. Similar results were obtained in 7 experiments. V_{sc}, which ranged between −96 and −108 mV in the period of hyperpolarization after incubation with 10 or 25 mM $[Li]_o$, was reduced

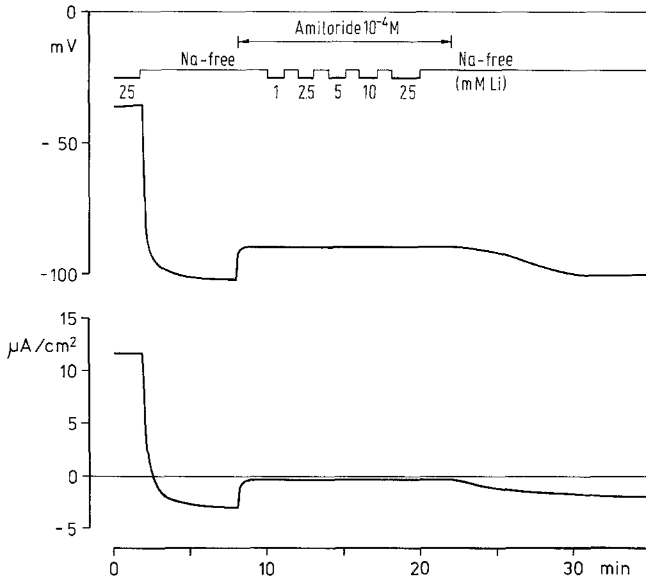

Fig. 2. Effect of amiloride (10^{-4} M) upon V_{sc} (upper panel) and I_{sc} (lower panel) of the frog skin after application of 25 mM Li and during addition of increasing [Li]. The epithelial surface was incubated with Na-free choline Ringer's solution in which Li substituted for choline on an equimolar basis during the indicated periods

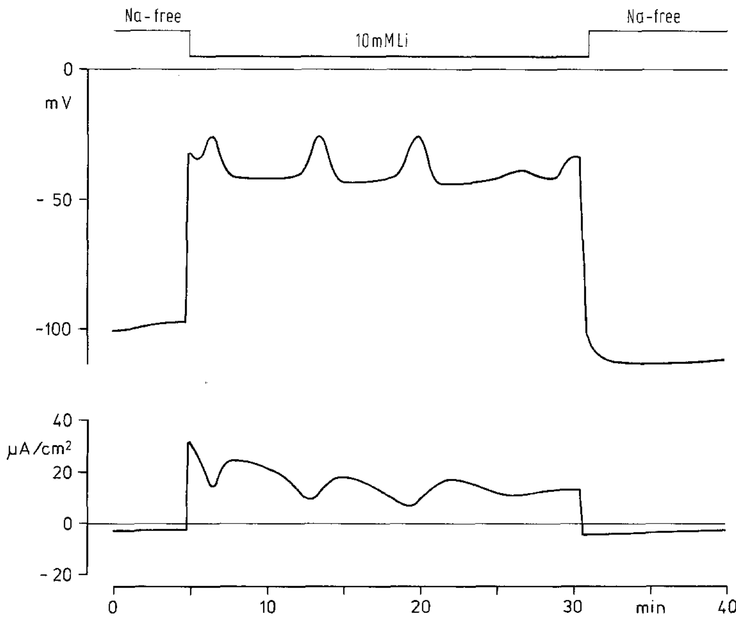

Fig. 3. Behavior of the V_{sc} (upper panel) and the I_{sc} (lower panel) upon addition of 10 mM Li (substituting choline) to the Na-free epithelial bathing solution

upon addition of amiloride to values between -85 and -94 mV. The I_{sc} was -3 to -7 μA/cm^2 before amiloride, but not significantly different from zero thereafter.

Amiloride, present in concentrations of 10^{-4} M in the epithelial bathing solution, prevented the depolarization of V_{sc} at all $[\mathrm{Li}]_o$. Fig. 2 shows a typical result. The change of V_{sc} was less than 1 mV even at $[\mathrm{Li}]_o = 25$ mM. In 4 experiments using 25 mM Li in the presence of Amiloride (10^{-4} M), the change of V_{sc} was always less than 3 mV and essentially no alteration of the I_{sc} was observed.

At 10 mM $[\mathrm{Li}]_o$, V_{sc} and I_{sc} showed peculiar transients. Fig. 3 demonstrates from a typical experiment, that these transients of 2–3 min duration occurred at regular intervals of 5–7 min during exposure to Li. The decrease of I_{sc} was associated with a depolarization of V_{sc}. During the periods between the transients, the intracellular potential remained almost at the value which was approached as a first steady state value 2–4 min after addition of 10 mM Li. The transients were also observed in some experiments with 5 mM $[\mathrm{Li}]_o$ and were always, but less clearly, present at 25 mM. At $[\mathrm{Li}]_o < 5$ mM, incubation up to 20 min did not produce transients of V_{sc} exceeding 3–4 mV.

A first stable value of V_{sc} was usually obtained 3–5 min after addition of Li. This value represents a reasonable measure of the steady state V_{sc} at the different $[\mathrm{Li}]_o$, since only slight and unsystematic changes of V_{sc} occurred thereafter. A plot of these potentials against the $[\mathrm{Li}]_o$ in a semilogarithmic scale is presented in Fig. 4. From a mean value of -93.6 ± 1.6 mV at Na-free, Li-free epithelial bathing solution, V_{sc} decreased to -73.0 ± 3.5 mV with 1 mM $[\mathrm{Li}]_o$. The further dependency of V_{sc} upon the $[\mathrm{Li}]_o$ can be described by a linear regression line with a slope of 35.6 mV per tenfold change of $[\mathrm{Li}]_o$ in the range between 1 and 25 mM $[\mathrm{Li}]_o$. Fig. 4 demonstrates that all individual experiments (connected by lines) fit remarkably well to the slope of the regression line (broken line). In four experiments, the incubation with the respective $[\mathrm{Li}]_o$ was extended for more than 10 min, i.e., until the final steady state potentials were recorded for several minutes. They are indicated by open circles in Fig. 4. No systematic difference is detectable between these values and those accepted from shorter periods of Li incubation.

Application of Li (20 or 50 mM) to the epithelial bathing solution in addition to 6 mM Na produced changes of the V_{sc} which are presented in Fig. 5 for a typical experiment. During the control period with 6 mM Na on the epithelial side, V_{sc} was -74 mV and the I_{sc} was 36 μA/cm^2. Addition of 20 mM Li to the epithelial bathing solution resulted in an

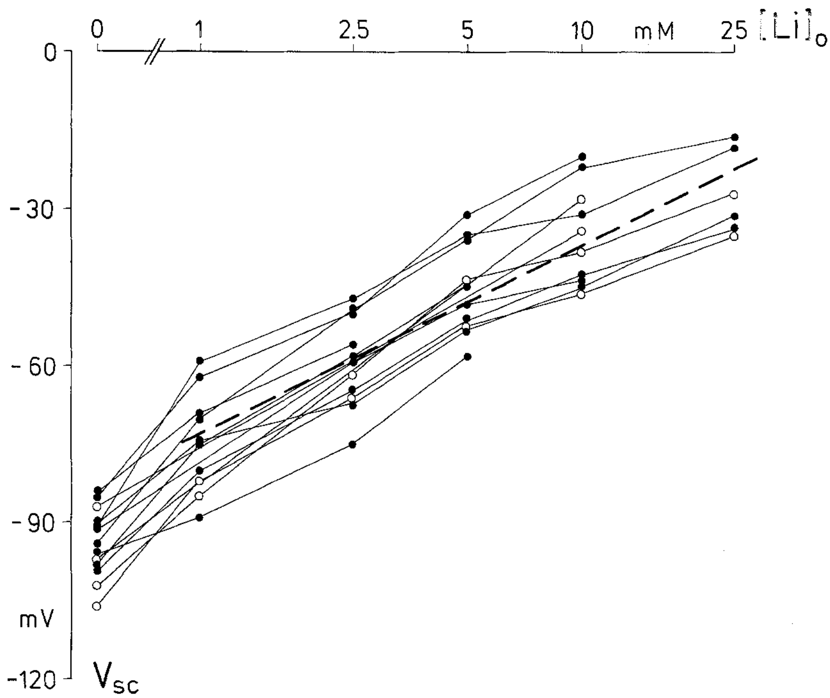

Fig. 4. Dependency of the intracellular potential under short circuit conditions (V_{sc}) upon the epithelial [Li] ($[Li]_o$). Individual experiments, i.e., measurements from the same cell, are connected by lines. Solid and open circles represent, respectively, short term (3–5 min) and long term (>10 min) incubation with the respective [Li]. The regression line with a slope of 35.6 mV/tenfold change of $[Li]_o$ was calculated for the range between 1 and 25 mM Li

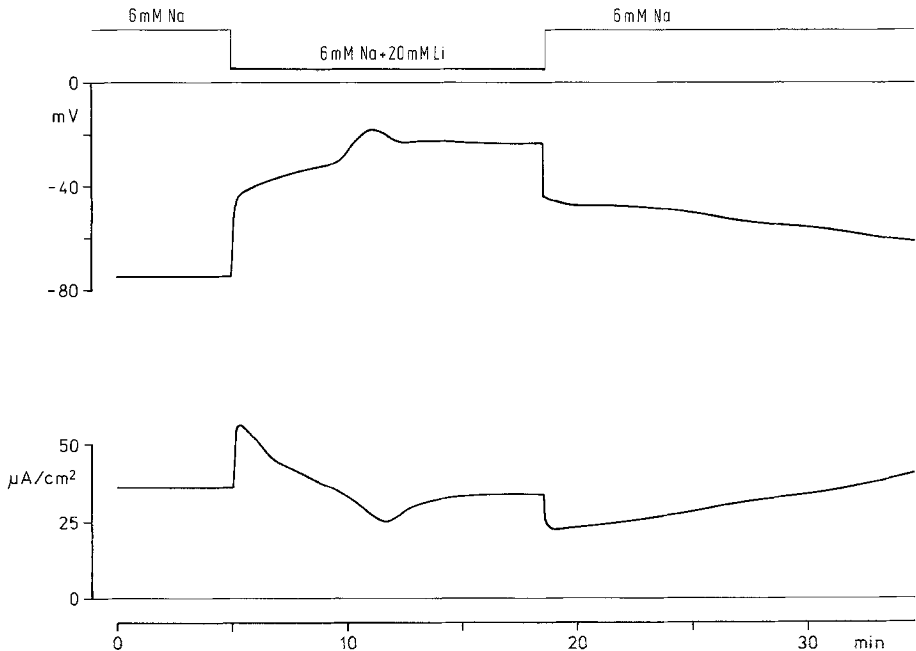

Fig. 5. Behavior of V_{sc} (upper panel) and I_{sc} (lower panel) upon addition of 20 mM Li (substituting Choline) when the epithelial surface was incubated with Choline Ringer's containing 6 mM Na

instantaneous decrease of V_{sc} until -42 mV followed by a slower approach to the steady state value of about -27 mV, which was reached 8 min after adding Li. The I_{sc} showed a considerable transitory increase, but decreased thereafter below control levels. Omission of Li from the epithelial side did not immediately lead to complete reversibility of the changes in V_{sc} and I_{sc}. A fast component of restoration was followed by a slow recline over several minutes.

In 7 experiments using 4 skins, the V_{sc} of -83 ± 3.8 mV in the presence of 6 mM Na at the epithelial side was reduced to -50.7 ± 5.4 mV 1 min after addition of 20 mM Li and to -34.6 ± 2.0 mV after approach to a steady state. Even larger changes in V_{sc} were observed when 50 mM Li was added to the epithelial bathing solution, but in view of the remarkable transitory changes of V_{sc} (and I_{sc}) no numerical evaluation was tried.

Discussion

The short circuited frog skin shows considerable intracellular electrical potentials even in the presence of [Li] up to 25 mM in the epithelial bathing solution. From the observed V_{sc}, equilibrium concentrations of Li in the intracellular space between 16 mM at $[Li]_o = 1$ mM ($[Li]_c/[Li]_o = 16$) and 60 mM at $[Li]_o = 25$ mM ($[Li]_c/[Li]_o = 2.5$) can be calculated for the short circuited frog skin. Passive uptake of Li across the outer border would be possible up to these concentrations. Under conditions similar to those of the present study, the ratio of $[Li]_c$ to $[Li]_o$ was found to be 10 at $[Li]_o = 1$ mM and 1.5 at $[Li]_o = 25$ mM (Leblanc, 1972). The $[Li]_c$ predicted from the intracellular potentials are always slightly higher than those observed by chemical analysis. This might result from differences of the experimental procedure since the isolated epithelium of *R. esculenta* was used for chemical analysis in contrast to the intact skin of *R. esculenta* in the present study. On the other hand, the difference might be explained by a certain loss of intracellular Li across the basolateral membranes towards the corial bathing solution. Transepthelial movement of Li has been demonstrated (Zerahn, 1955; Candia & Chiarandini, 1973; Reinach *et al.*, 1975), and at least part of this was suggested to occur across the cellular pathway. Then, the maximal $[Li]_c$, predicted from the Nernst equation, must be reduced in relation to the, yet unknown, permeability of the inner membrane.

A different explanation for the accumulation of Li in the frog skin epithelium was forwarded from analysis of the current voltage curve of the outer membrane in the presence of Li (Cirne & Lindemann,

1975). From the constant field equation, the cellular Li activity was calculated to approach, but never to exceed, the outer Li activity. These authors conclude that the Li accumulation "does not occur in the transport compartment..., but in another compartment in series to this one". However, since the experiments were performed in K-depolarized skins, it must be expected from the present analysis that, in the absence of intracellular negative electrical potentials, Li does not accumulate in the intracellular space. Then, no necessity exists to postulate additional compartments in series to the transport compartment which are responsible for Li accumulation.

In this context it should be mentioned that incubation with isotonic K-Ringer's at the corial side does not completely depolarize the basolateral membranes (Nagel, 1977*a*). If negative intracellular potential under short circuit conditions would exist also in the presence of low concentrations of Li in the outer solution, this might explain why Li is accumulated even in K-depolarized skins (Morel & Leblanc, 1975). The above conclusion regarding the experiments of Cirne and Lindemann (1975) would, however, not be influenced, since their computations are based upon the presumption that the inner (and outer) membrane potential is zero. If this is incorrect, the calculation of absolute numerical values for the intracellular Li activity is not valid.

The striking accordance between the results of previous chemical determinations (Leblanc, 1972) and the predictions from the present electrical measurements demonstrates that passive uptake of Li across the outer border of the epithelial cells is responsible for the accumulation of Li in the epithelium. No necessity exists to postulate active transport of Li at the outer border (Leblanc, 1972), which, however, was based upon previous measurements of the intracellular potential (Biber & Curran, 1970), which were most likely obtained after large injury of the punctured cells. Similar observations to those reported in the literature, i.e., constancy of the microelectrode potentials at values of about -15 to -20 mV, could also be obtained in the present investigation, but only after the microelectrode potentials showed a breakdown indicating cell injury by the microelectrode (Nagel, 1976*b*). Values after a breakdown of the intracellular potential were not accepted in the present study. The potentials observed in the present investigation are slightly less negative than those reported previously (Helman & Fisher, 1977; Nagel, 1977*b*). Seasonal variations and the fact that Na-free solution at the epithelial surface was applied for long periods (more than 20 min) might explain the difference. This could abolish the influence of electrogenic components of the Na pump (Nagel, 1976*b*; Nagel & Helman, 1977)

and lead to intracellular potentials near the potassium equilibrium potential. It should be mentioned that the recorded intracellular potentials were, generally, not obtained from the first cell layer, although only indirect evidence can be given for this suggestion. In most punctures, the accepted intracellular potentials were recorded after the impalement and loss of a preceeding cell layer. Nevertheless, since the cells of frog skin epithelium are electrically coupled (Nagel, 1976*c*), the real potential gradient across the outer border is obtained.

The dependency of the intracellular potentials upon the [Li] in the epithelial bathing solution can be described by a semilogarithmic linear relationship with a slope of 36.5 mV per tenfold change of $[Li]_o$. This might be interpreted as an indication of a Li electrode function existing at the Li permeable outer border of the frog skin (Lindley & Hoshiko, 1964), as had been proposed for Na at this membrane (Koefoed-Johnson & Ussing, 1958). Experimental results which raise doubt regarding this explanation for the case of Na are discussed elsewhere (Nagel, 1976*a*, 1977*b*). The semilogarithmic linear correlation between V_{sc} and $[Li]_o$, however, clearly does not represent a Li electrode function. This follows from the estimation of the [Li] gradients between the outer solution and the cellular space. For example, at $[Li]_o = 2.5$ mM, the final steady state $[Li]_c$ of about 15 mM is approached within ≈ 30 min (Leblanc, 1972). If Li electrode like potentials would contribute significantly to the generation of V_{sc}, there should be a continuous change of V_{sc} during this period. The present results demonstrate that such changes of V_{sc} are missing up to 25 min after addition of Li. Especially at low $[Li]_o$, which most likely does not lead to secondary disturbances of the system, the steady state V_{sc} is reached within 10–15 sec and remains essentially constant thereafter. Further on, this observation rules out that alterations of the K dependent potential difference of the basolateral membranes (Koefoed-Johnson & Ussing, 1958; Lindley & Hoshiko, 1964; Helman & Fisher, *personal communication*; Nagel *unpublished observation*) are responsible for the change in V_{sc}. Although a reduction of the intracellular [K] is likely to occur when Li accumulates in the cells, this should result in a time-dependent change of V_{sc} which, clearly, was not observed. A more feasible explanation for the experimental data would be that Li enters the intracellular space primarily driven by the electrical gradient which is generated at the basolateral membranes. This influx of positively charged ions necessarily depends upon the $[Li]_o$ and must result in a gradual depolarization of the intracellular potential due to the $R \cdot I$ drop of this current. The linearity of the semilogarithmic plot between $[Li]_o$ and V_{sc} would then be fortuitous and without meaning for the mechanism

of membrane passage. A similar explanation has been proposed for the apparent Na electrode-like behavior of the intracellular potentials upon change of the epithelial [Na] (Nagel, 1976*a*, 1977*b*).

The hypothesis of current-dependent changes of the intracellular potential is supported by the observation that the V_{sc} after exposure to Li, when Na-free choline Ringer's incubates the epithelial surface, shows a temporary hyperpolarization, which depends upon the previous $[Li]_o$ and the duration of Li incubation. Addition of amiloride to the choline Ringer's solution results in immediate depolarization of the V_{sc} to values near the previous control values. Amiloride was demonstrated to inhibit the Li efflux from the preloaded frog skin epithelium (Morel & Leblanc, 1975). Hyperpolarization and amiloride sensitivity of this potential change are easily explained as resulting from Li currents with the appropriate $R \cdot I$ drop in the outward direction.

The result, that amiloride (10^{-4} M) in the epithelial bathing solution prevents the change of V_{sc} by Li almost completely, is not surprising. Amiloride, which blocks Na permeation through the outer border (Eigler, Kelter & Renner, 1967; Bentley, 1968; Dörge & Nagel, 1970; Rick, Dörge & Nagel, 1975), has the same inhibitory effect upon Li movements (Leblanc, 1972; Candia & Chiarandini, 1973; Morel & Leblanc, 1975; Reinach *et al.* 1975). The microelectrode data demonstrate, in accordance with previous results (Morel & Leblanc, 1975), that both, Li influx and efflux across the outer border, are amiloride sensitive and point to a common pathway for the two unidirectional fluxes. Morel & Leblanc (1975) were able to show similar effects of amiloride upon the Na influx and efflux. In microelectrode studies no effect of amiloride upon possibly existing Na efflux is detectable (my *unpublished observation*). However, the intracellular concentration of Na which is actively transported out of the cells, is maintained at comparatively low values of 10–20 mM (Zylber, Rottuno & Cereijido, 1973; Aceves & Erlij, 1971; Dörge, Gehring, Nagel & Thurau, 1974). Consequently, the amount of Na leaving the cellular space towards the epithelial bathing solution against the electrical gradient of more than 90 mV might be too small to generate a measurable $R \cdot I$ drop. In this context it must be considered that Morel and Leblanc (1975) used K-depolarized frog skins which have considerably reduced intracellular potentials in the short circuited state (Nagel, 1977*a*). Then the efflux of Na to the epithelial side may be increased.

Peculiar transistory changes of the V_{sc} and the I_{sc} were observed at $[Li]_o > 5$ mM. These transients are comparable to those reported previously from transepithelial measurements (Teorell, 1954; Thellier *et al.*,

1976). The present data cannot give information regarding the participation of the inner or outer membrane in the generation of these potential and current changes. Preliminary results, however, show that the inner border electromotive force and resistance are reduced during the period of depolarization. Experiments to further characterize these effects of Li, which may in addition provide insights into the mechanism of Li transport across the basolateral membrane, are in progress.

The transepithelial movement of Li has been used as a tool to investigate the mechanism of transepithelial Na transport (Hansen & Zerahn, 1964; Biber & Curran, 1970; Leblanc, 1972; Candia & Chiarandini, 1973; Reinach *et al.*, 1975). Until now, however, the magnitude and behavior of the electrical gradients across the epithelial cell membranes could not be adequately considered. According to the present findings, the uptake of Li at the outer border can readily be explained by passive processes and does not require an active transport step. Consequently, the assumption of active Na transport at the outer border of the frog skin, based upon the similarities between transport of Li and Na, cannot be supported from this point of view. Indeed, the entry of Na across the outer border is favored by the electrical gradient under all conditions and it can be demonstrated that passive Na uptake can occur even at epithelial [Na] below 1 mM (Helman & Fisher, 1977; Nagel, 1977*b*).

The observation of interference between the fluxes of Li and Na at the outer border of frog skin has been suggested to reflect competition of the two cations for a common carrier (Biber & Curran, 1970). Since considerable changes of the intracellular potential are induced by Li, the hypothesis of competition of Li and Na for a common carrier requires re-examination. Under experimental conditions, similar to those of the study by Biber and Curran (1970), V_{sc} is found to be reduced in the presence of 20 mM Li by more than 40 mV. Biber and Saunders (1973) demonstrated that the Na uptake across the outer border of the frog skin is very sensitive to changes of potential. From the data (Table II of Biber & Sanders (1973)) it can be calculated that a change of the potential difference across the outer border by 40 mV results in a reduction of Na uptake by at least 30%[1]. The observed reduction of the

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1 For the calculation it is assumed that the transepithelially applied voltage clamping would affect only the outer membrane. This, however, is a minimal estimate since at 6 mM Na in the epithelial bathing solution the fractional resistance of the outer border is in the range of 0.8 to 0.9 of the total resistance (my *unpublished results*). The change of V_{sc} by $\sim$40 mV would then correspond to transepithelial voltage clamping to 45–50 mV and the influence upon the Na uptake would be even higher.

Na uptake in the presence of 20 mM Li was found to be about 20% (Table IV of Biber and Curran (1970)) and can easily be explained by the voltage dependency of the Na uptake mechanism. Although the present investigation cannot rule out or in, whether carrier mediation participates in the transport of Na across the outer border of the frog skin epithelium, the data demonstrate that a supposed argument in favour of carrier mediation, i.e., competitive inhibition of the Na flux by Li, cannot be supported since potential changes must account for this observation. Further experimental work is necessary to provide more direct insights into the mechanism of Na transport across the outer border.

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