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Cellular Li^+ Opens Paracellular Path in Toad Skin: Amiloride Blockable Effect

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Summary. The presence of Li in the solution bathing the outer surface of toad skin under short-circuit condition promotes an unspecific permeability increase characterized by a delayed and progressive increase in the effluxes of ^{24}Na, ^{42}K and ^{14}C sucrose. The effect of Li upon sucrose permeability might indicate an increased permeability of the paracellular pathway. The Li effect is mediated by an intracellular action since blockade of Li entrance into the cell compartment by amiloride prevents the increase in Na, K and sucrose permeability. A possible mechanism of this effect is discussed in terms of a disturbance in the cellular Ca^{++} balance leading to an increase in cytosolic Ca^{++} concentration which perturbs the organization of the cytoskeleton and the interplay between cytoskeleton and tight junctions.

Key Words toad skin · paracellular pathway · lithium · amiloride · cytosolic Ca^{++}

Introduction

There are many reports in the literature dealing with the effect of Li upon amphibian epithelial membranes [2, 3, 5, 6, 14, 15, 17–19, 21, 22, 24, 26, 28, 36, 38]. In frog skin, Li easily crosses the apical barrier of the epithelial cells [2, 21, 24]. However, the role of the Na, K-ATPase on the mechanism of Li extrusion across the basolateral membrane is still controversial [19, 32, 33]. Several effects of Li upon epithelial membranes have been described: inhibition of the Na transport, and of the natriferic response to vasopressin and cyclic AMP in toad bladder [3]; decrease of Na transport and SCC in frog skin [38]; possible inhibition of renal adenyl cyclase [3, 8]; a less effective feedback inhibition of apical Na channels by its accumulation in the cell compartment, as compared to Na accumulation [21].

The initial purpose of the present study was to use Li as a Na substitute in the outer solution, as a way to mimic, at least in a first approach, the effect of ouabain blocking the Na-pump upon the effluxes of ^{42}K and ^{24}Na in toad skin [4, 37]. The rationale underlying these experiments is that Li being not effectively pumped [31, 33] and also inhibiting the Na, K-ATPase activity [6] would act as blocking this enzyme and would simulate the effect of ouabain upon the active Na transport. However, our experiments gave a striking and unexpected result. Li had a marked effect increasing the permeability of the paracellular pathway due to an intracellular action that could be prevented by blocking its entrance into the cell compartment by amiloride.

Materials and Methods

These studies were carried out in modified Ussing-Zerahn chambers, according to methods previously described (4, 36, 37). Special precautions were taken to prevent the effect of skin edge damage on the low levels of isotope efflux by using hemichambers provided with a silicone grease gasket (High Vacuum Grease) located at the internal rim of the hemichamber surface in contact with the epithelial side of the skin. A nylon mesh in contact with the corial side was used to prevent lateral skin movement during changes of solution in the outer compartment.

Abdominal skins of the toad *Bufo marinus ictericus* were used and the experiments were performed in the short-circuited state at room temperature (20 to 25 °C). A voltage-clamp unit was connected to the preparation through saturated 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 h or more, according to the protocol of each experimental group (as will be referred to in Results), elapsed before the addition of 100 μCi of ^{24}Na or ^{42}K (Institute of Energetic and Nuclear Research, São Paulo, Brazil) or 25 μCi of ^{14}C sucrose (New England Nuclear, Boston, Mass.) to the solution bathing the inner skin surface (corial side). Every 5 min, the outer compartment solution was completely drained into counting vials for ^{24}Na or ^{42}K assay by the Cerenkov effect [25], in a liquid scintillation counter (Beckman, Mod. LS-8000). ^{14}C sucrose was assayed in the same equipment in aliquots of 200 μl taken from the solution drained from the outer compartment. Short-circuit condition was maintained throughout the experiments, except for 5 to 10 s during drainage of the outer compartment. J_{eff}^{Na}, J_{eff}^{K} and J_{eff}^{suc} are the

effluxes of Na, K and sucrose calculated from the rate of appearance of the respective isotopes in the outer compartment and the corresponding specific activity in the inner bathing solution.

Solutions used were (in mM): NaCl-Ringer's solution – NaCl 115.0, $KHCO_3$ 2.5, $CaCl_2$ 1.0; 10% Li-NaCl-Ringer's solution-NaCl 103.5, LiCl 11.5, $KHCO_3$ 2.5, $CaCl_2$ 1.0; KCl-Ringer's solution-KCl 115.0, $KHCO_3$ 2.5, $CaCl_2$ 1.0; 10% Li-KCl-Ringer's solution-KCl 103.5, LiCl 11.5, $KHCO_3$ 2.5, $CaCl_2$ 1.0; LiCl-Ringer's solution-LiCl 115.0, $KHCO_3$ 2.5, $CaCl_2$ 1.0; All solutions had pH 8.2 after aeration. Drugs used were: ouabain from Sigma Chemical Company and amiloride from Merck Sharp & Dohme Research Laboratories.

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

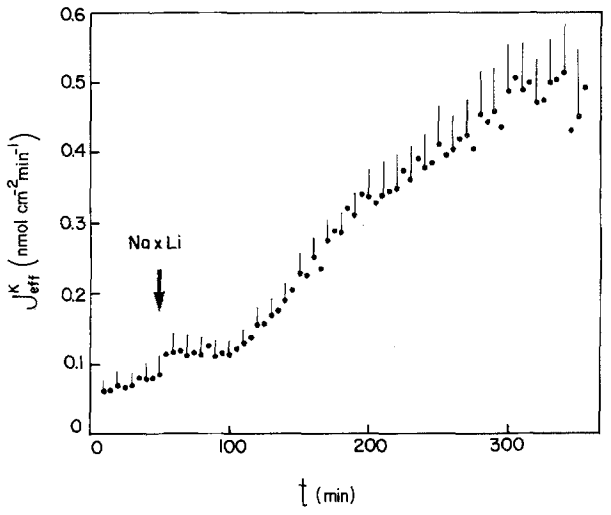

Fig. 1. Effect of substitution of NaCl-Ringer's solution by LiCl-Ringer's solution in the outer compartment upon the steady-state rate of ^{42}K discharge into the external compartment (J^K_{eff}). Inner bathing medium was NaCl-Ringer's solution. ($n=12$)

Results

Effect of Na by Li Replacement in the Outer Bathing Medium Upon the K Efflux (J^K_{eff})

These experiments were performed with NaCl-Ringer's solution initially bathing both sides of the skin. After J^K_{eff} had reached a steady state, normally within 100 min after addition of the isotope to the inner compartment, approximately 10 control determinations of J^K_{eff} were carried out. Then the outer bathing medium was replaced by LiCl-Ringer's solution and the collections followed as described in Materials and Methods. Figure 1 shows the behavior of J^K_{eff} in the control and in the presence of Li. As seen, after a delay of approximately 30 to 40 min, a steady increase of J^K_{eff} with time was observed and no plateau was reached within the experimental period. At the end of the experiment a 4.6-fold increase was observed in J^K_{eff} in relation to the control situation.

As control experiments, we studied the temporal stability of J^K_{eff} in skins bathed for 5 h with NaCl-Ringer's solution on both sides, and also the effect of Na withdrawal from the outer compartment and its substitution by K upon J^K_{eff}. Figure 2 shows the results. As can be seen, J^K_{eff} is very stable with time for more than 200 min when skins are bathed by NaCl-Ringer's solution on both surfaces. When Na is totally replaced by K in the outer bathing medium no increase in J^K_{eff} is observed, in sharp contrast with the results of Li substitution experiments.

In a group of three experiments, after 2.5 h of Li being in contact with the external skin surface,

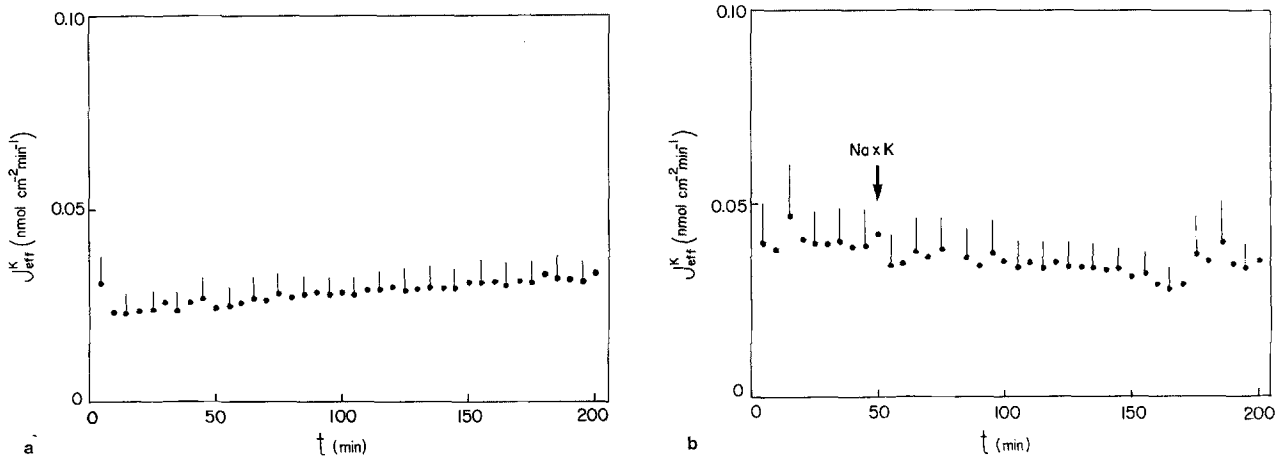

Fig. 2. *a*) Temporal stability of the rate of ^{42}K discharge into the external medium (J^K_{eff}) when outer and inner solutions were NaCl-Ringer's solution throughout the experiment ($n=4$). *b*) Absence of effect of substitution of NaCl-Ringer's solution by KCl-Ringer's solution in the outer bathing medium upon J^K_{eff}. Inner solution was NaCl-Ringer's. ($n=7$)

the outer bathing solution was again replaced by NaCl-Ringer's solution and no recuperation was observed for the subsequent 100-min period, except for a J^K_{eff} tendency to reach a plateau.

Further tests for the Li effect upon J^K_{eff} and for the reversibility of the phenomenon were carried out by partial Na by Li substitution in the outer compartment in two different conditions: in one group, NaCl-Ringer's solution was replaced by 10% Li-NaCl-Ringer's solution. This did not induce any increase in J^K_{eff}. A slight decrease of J^K_{eff} (in nmol cm^{-2} min^{-1}) from (0.604 ± 0.014) 10^{-1} in the control condition to (0.504 ± 0.014) 10^{-1} after the ionic substitution was indeed observed. J^K_{eff} value after returning to the control condition was (0.529 ± 0.004) 10^{-1} nmol cm^{-2} min^{-1}. In another group of experiments, the control situation was KCl-Ringer's solution in the outer compartment and NaCl-Ringer's solution in the inner compartment. Substitution of the outer solution by 10% Li-KCl-Ringer's solution, in contrast to the results of the previous group, induced, after a delay of approximately 50 min, an increase in J^K_{eff} which was perfectly reversible upon return to the control KCl-Ringer's solution, as shown in Fig. 3. It is worth noting that the time course of J^K_{eff} increase upon the presence of 10% Li in the outer compartment is much slower than the reversal effect induced by Li withdrawal from this compartment.

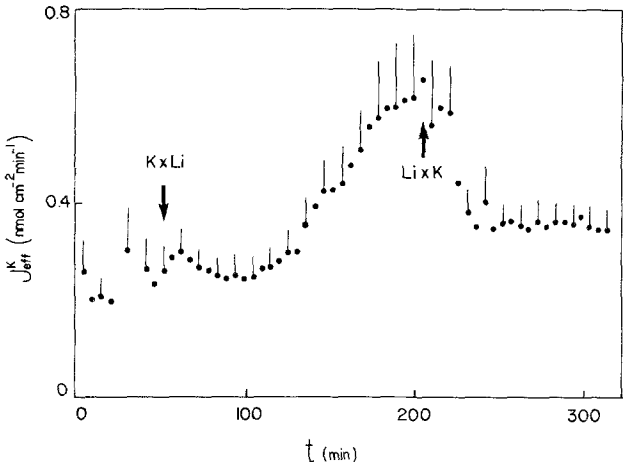

Fig. 3. Effect of substitution of KCl-Ringer's solution by a solution containing 10% of Li replacing equimolar amount of K in the outer compartment upon the rate of ^{42}K discharge into the outer medium (J^K_{eff}) (first arrow). At the second arrow the outer bathing medium was returned to the control KCl-Ringer's solution. Inner bathing medium was NaCl-Ringer's solution. ($n=8$)

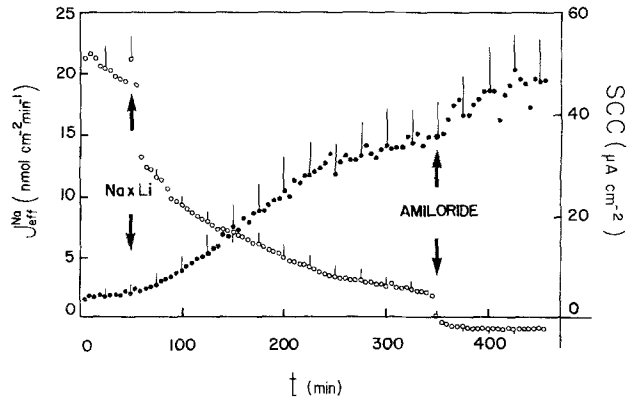

Fig. 4. Effect of substitution of NaCl-Ringer's solution by LiCl-Ringer's solution in the outer compartment upon the rate of ^{24}Na discharge into the external medium (J^{Na}_{eff}) (●) and the short-circuit current (SCC) (○). Amiloride 10^{-4} M was added when J^{Na}_{eff} had reached a steady state. Inner solution was NaCl-Ringer's solution. ($n=9$)

Effect of Na by Li Replacement in the Outer Bathing Medium Upon the Na Efflux (J^{Na}_{eff})

These experiments were performed in conditions similar to those already described for J^K_{eff}.

With NaCl-Ringer's solution on both sides of skin and J^{Na}_{eff} in steady state, total Na by Li substitution in the outer solution induced a steady increase of J^{Na}_{eff} with time, reaching a plateau 250 min after Li replacement (Fig. 4). The increase starts almost immediately after the ionic substitution in the outer compartment. The plateau value is 8.2-fold higher than the control J^{Na}_{eff}. At this stage, addition of amiloride (10^{-4} M) to the outer compartment induced a slight increase of J^{Na}_{eff} simultaneously to a sharp decrease of the SCC which was already low as a consequence of the Na by Li replacement (Fig. 4).

As for J^K_{eff}, the effect of total Na by Li substitution upon J^{Na}_{eff} is not reversible.

A different group of experiments was performed with skins previously treated with ouabain in order to completely block the rate of transepithelial Na transport and to lead to a condition of high cellular Na concentration [29]. This condition is known to induce a large reduction of Na permeability of the outer barrier through a feedback mechanism of Na self-inhibiting its own channels in this structure [4, 11]. When sufficient time had elapsed after addition of ouabain for the transient increase of J^{Na}_{eff} induced by this inhibitor [4] to be overcome, total Na by Li replacement was carried out in the outer compartment. Figure 5 shows that J^{Na}_{eff} still displays a small tendency to

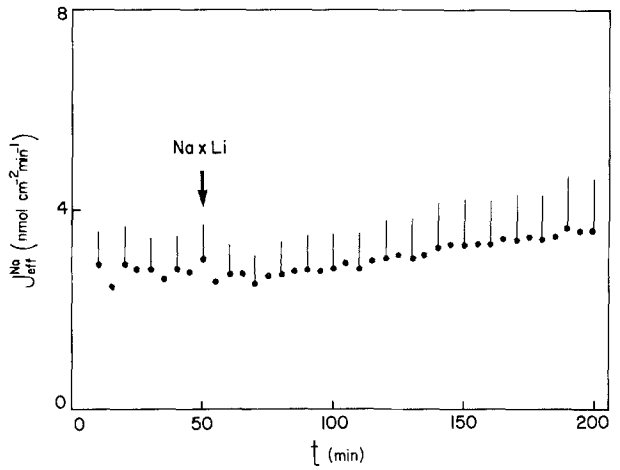

Fig. 5. Effect of substitution of NaCl-Ringer's solution by LiCl-Ringer's solution in the outer compartment upon the steady-state rate of ^{24}Na discharge into the external medium (J_{eff}^{Na}) in skins treated with ouabain 10^{-3} M. Inner solution was NaCl-Ringer's solution. Ouabain was added to that solution 3.5 h before the ionic substitution in the outer compartment. ($n=4$)

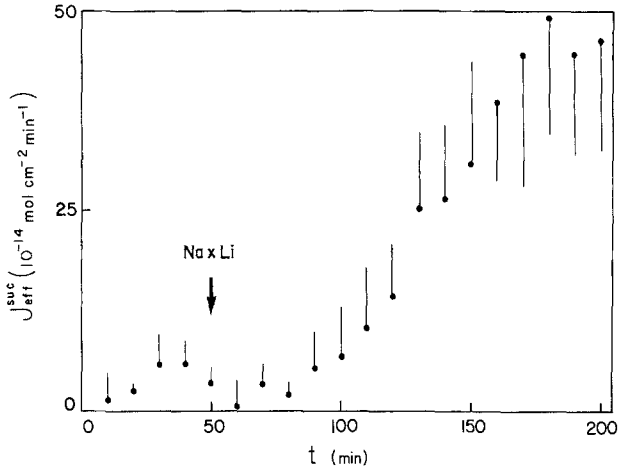

Fig. 6. Effect of substitution of NaCl-Ringer's solution by LiCl-Ringer's solution in the outer compartment upon the steady-state rate of ^{14}C sucrose discharge into the external medium (J_{eff}^{suc}). Inner solution was NaCl-Ringer's solution ($n=4$)

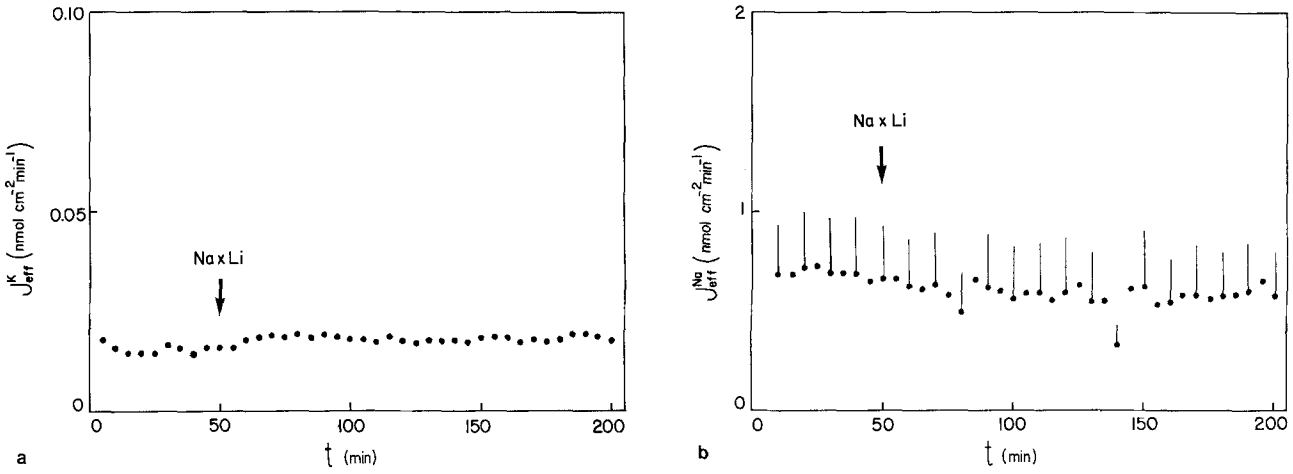

Fig. 7. Effect of substitution of NaCl-Ringer's solution + amiloride 10^{-4} M by LiCl-Ringer's solution + amiloride 10^{-4} M in the outer compartment upon the steady-state rates of: *a*) ^{42}K discharge into the external medium (J_{eff}^{K}) ($n=5$) (the error bars are within the size of the dots); *b*) ^{24}Na discharge into the external medium (J_{eff}^{Na}) ($n=7$). Inner medium was in both cases NaCl-Ringer's solution

increase but at a much smaller rate than seen in Fig. 4 for skins not treated with ouabain.

Effect of Na by Li Replacement in the Outer Bathing Medium Upon the Sucrose Efflux (J_{eff}^{suc})

These experiments were performed in order to look for possible changes in permeability of the paracellular pathway induced by the presence of Li in the outer compartment.

25 μCi of ^{14}C sucrose was added to the inner bathing medium of skins bathed on both sides by NaCl-Ringer's solution, following the same experimental protocol used for determinations of J_{eff}^{K} and J_{eff}^{Na}.

In the control condition with NaCl-Ringer's solution on both sides of skin the rate of ^{14}C sucrose efflux was stable and low. Total Na by Li substitution in the outer compartment induced, after a delay of 30 min, a steady increase of J_{eff}^{suc} (Fig. 6) following a pattern similar to that already seen for J_{eff}^{K} (Fig. 1). The reversibility of the effect of Li upon J_{eff}^{suc} was not tested.

Protection by Amiloride of the Li Effect Upon J_{eff}^{suc}, J_{eff}^{Na} and J_{eff}^{K}

These experiments were carried out with skins bathed by NaCl-Ringer's solution on both sides. Total Na by Li substitution was performed in the outer compartment. Amiloride 10^{-4} M was present in the outer compartment throughout the whole experiment.

J_{eff}^{suc} was barely detectable in the control as well as in the test period with Li plus amiloride in the outer medium, the values fluctuating around the blank value for 4 skins. J_{eff}^{K} and J_{eff}^{Na} did not change after the Na by Li substitution in the presence of amiloride as can be seen in Fig. 7.

Discussion

The results of the present work indicate that the presence of Li in the solution bathing the outer skin surface leads to an unspecific skin permeability increase which is characterized by a progressive increase of the effluxes of ^{14}C sucrose, ^{42}K and ^{24}Na, as shown in Figs. 1, 4 and 5. This Li effect is mediated by an intracellular action, since blockade of Li entrance into the cell compartment by amiloride prevents permeability increase to Na and K (Figs. 7*a* and 7*b*) and to sucrose.

Li specificity to increase skin permeability to ^{42}K was tested in order to distinguish it from a possible effect due to Na withdrawal from the outer medium. Figure 2*b* shows that Na withdrawal from that medium and its substitution by K does not induce increase of ^{42}K efflux, which remains at a stable level throughout the experimental period of approximately 150 min. In a control group, the temporal stability of J_{eff}^{K} was also tested with NaCl-Ringer's solution on both sides of skin. This was done in order to discard the possibility that the increase observed in the effluxes of ^{42}K, ^{24}Na and ^{14}C sucrose, following the presence of external Li, was due to a temporal skin deterioration. However, Fig. 2*a* clearly shows that skin permeability is a stable parameter when the preparation is bathed by NaCl-Ringer's solution on both sides. Therefore, these results support the interpretation that the increase in skin permeability is a specific action of Li ions.

The reversibility of the Li effect was tested in experiments where Na in the outer solution was totally replaced by Li, and after 150 min the control NaCl-Ringer's solution was again replaced in the outer compartment. Lack of reversibility was observed both for ^{42}K and ^{24}Na effluxes which remained high. For the ^{42}K experimental group, the return to the control condition led to an apparent stabilization of J_{eff}^{K}. The effect of partial Na by Li substitution upon J_{eff}^{K} and J_{eff}^{Na} and their reversibility were also studied. The results indicate that the equimolar 10% Na by Li substitution in the outer medium apparently is not sufficient to trigger skin permeability increase as is observed when Na is totally replaced by Li. There are several evidences in literature indicating that Na and Li share common pathways or mechanisms in amphibian skins [5, 19, 24]. Therefore, having in mind that Na could compete with Li ions for a given locus or mechanism blocking the Li effect, experiments were carried out in the absence of external Na, with KCl-Ringer's solution as outer bathing medium. As shown in Fig. 3, 10% K by 10% Li substitution in the outer medium presented a marked effect inducing an increase of ^{42}K permeability, which displayed the same characteristics already presented in Fig. 1, except for being reversible when Li was removed from the outer compartment and replaced by K.

The increase of ^{24}Na efflux induced by Li apparently does not occur via amiloride-sensitive Na channels of the apical barrier. In fact, the use of this inhibitor did not cause any reduction in J_{eff}^{Na} after this efflux had already been enhanced by the Li effect. On the other hand, short-circuit current previously depressed by the presence of Li in the outer medium was promptly and further reduced by amiloride. It is interesting to notice that amiloride indeed induced a small but clear increase of J_{eff}^{Na} as shown in Fig. 4, in contrast to its known effect as a Na channel blocker at the apical barrier [9]. So far, we have no reasonable explanation for this amiloride effect. A paradoxical effect of amiloride was also described [4] when this inhibitor was tested upon the ^{24}Na efflux in toad skins with the apical Na channels already blocked by a high cell Na concentration. The increased Na efflux induced by Li, being not amiloride-sensitive, was interpreted as due to a paracellular permeability increase. Apparently, this seems to be the explanation for the skin permeability alteration induced by Li since this ion leads to an increase of ^{14}C sucrose efflux, sucrose being a known probe of the paracellular pathways.

Other known maneuvers leading to tight junctions opening in tight epithelia are hypertonicity [10, 13, 34, 35] and acidification [1, 12] of the outer bathing solution. The time courses of these effects are normally much faster than those induced by Li upon J_{eff}^{K} and J_{eff}^{suc}. For the acidification of the outer bathing solution [1] the effect could already be clearly detected within the first 5 min upon addition of acid to the external medi-

um. The same could be said for the onset of the hypertonicity effect [10]. These effects are currently assumed to be due to an extracellular action at the outer skin surface. For the case of acidification, it seems that the locus for the hydrogen ion effect is not easily accessible from the outer solution since H_2SO_4 at pH 2.1 is much less effective than HCl at the same pH. This difference was interpreted [1] as being due to the existence of a diffusion potential possibly arising at the structure of the tight junction itself due to a lower SO_4^{2-} mobility as compared to that of Cl^- ion. However, the possibility of an intracellular action of H^+ ion cannot be discarded.

As Li easily crosses the apical barrier through the Na channels, reaching the cell compartment [19, 24], it is advisable to be able to distinguish between an extracellular and an intracellular effect of this ion-inducing tight junction opening. The use of amiloride, a powerful blocker of apical Na channels [9, 27] which also blocks Li movement in these channels [9, 24] proved to be a powerful tool for deciding between those two possibilities. Amiloride-treated skins displayed a clear contrast in relation to the control ones since Na by Li substitution in the outer bathing solution was unable to elicit skin permeability increase to ^{24}Na, ^{42}K and ^{14}C sucrose.

The experiments performed in ouabain long-term treated skins also support an intracellular action of Li ions. The much milder effect (Fig. 5) of total Na by Li substitution in the outer solution in these skins as compared to the control ones, not treated with ouabain, could reflect a much lower apical membrane permeability to Na and also to Li, a consequence of Na-self inhibition upon its own channels in the apical barrier due to a high cellular Na concentration induced by ouabain poisoning [4, 11, 20].

So, the results mentioned above permit us to conclude that the Li effect increasing skin permeability is due to an action upon intracellular structures.

A reasonable hypothesis concerning the Li effect upon the tight junctions could be put forward on the basis of data in literature which suggest that the cytosolic Ca^{++} concentration might play a role as a mediator in the process. Lithium has been reported as antagonizing the actions of calcium in several preparations [16]. In salivary glands of *Chironomus* it has been shown [30] that substitution of extracellular Na by Li causes depression of gap junction permeability with a response which resembles our results regarding latence of onset, irreversibility and Li-specificity. The authors interpreted this effect on the grounds of an increase in cytosolic Ca^{++} concentration induced by Li. Li may influence carbohydrate metabolism at various levels due to an inhibitory effect upon enzymes involved in the carbohydrate metabolism [23] and in this way might disturb the cellular Ca^{++} balance leading to an increase in cytosolic Ca^{++} concentration. This would obviously perturb several cell systems, namely the cytoskeleton and the interplay between cytoskeleton and tight junctions. In fact, a clear interrelationship between cytosolic Ca^{++} concentration, microfilaments and opening or sealing of tight junctions in MDCK monolayers has been shown [7]. The tight junctions open when the cytosolic Ca^{++} concentration is increased and the structural components of the cytoskeleton, particularly the microfilaments, seem to be involved in this junctional event.

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