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Nonhormonal Mechanisms for the Regulation of Transepithelial Sodium Transport: The Roles of Surface Potential and Cell Calcium

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Summary. An attempt to define the main categories of regulatory mechanisms of transepithelial sodium transport across tight epithelia is presented.

In particular, evidence suggesting two types of mechanisms, changes in surface potential and the level of cell Ca, are described in greater detail.

We have measured the effects of conditions that affect surface potential on the transepithelial sodium transport. Those conditions that increase the screening of negative charge and therefore depolarize the outer membrane are expected to have effects homologous to a depolarization caused by external current. Indeed, when the composition of the outside solution was modified by (i) increasing ionic strength, (ii) adding polyvalent cations (La^{+++}, Co^{++}, Ni^{++}, Cd^{++}), or (iii) lowering pH, an increase in active Na transport was detected. Moreover, the presence of small concentrations of polyvalent cations which screen surface charge, markedly dampens or even eliminates the effects of pH or ionic strength on Na transport. These findings provide additional support for the notion that a potential-sensitive component regulates Na movements across the apical membrane of the frog skin, and offer a framework to understand the effects of numerous cationic agents on transepithelial transport that hitherto remain unexplained.

With respect to the role of intracellular Ca we have found that procedures that increase cell Ca, like removal of sodium in the basal solution or addition of ionophore A23187, reduce transepithelial Na transport. Moreover, conditions that block the increase in cell Ca prevent the inhibition of transport. These observations suggest that the level of intracellular Ca may determine the rate of transepithelial Na transport.

One of the most fascinating features of transepithelial transport of Na is that it can vary over a wide range of values in the same piece of tissue. Such a broad range of operation is not only of interest because it provides a mechanism for the regulation of salt metabolism in the

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Table 1. Mechanisms for the regulation of Na movements across tight epithelia

I. Hormones	Antidiuretic hormone Aldosterone
II. Potential	Transmembrane potential Surface potential
III. Intracellular ion concentration	Ca^{++} Na^{+}

whole organism, but also because it may provide a tool to understanding the organization of the transport system.

The main regulatory mechanisms of tight epithelial function appear to be localized at the Na entry step of the apical barrier. In Table 1 we have summarized the mechanisms that we consider to be involved in this process. On the one hand there are the hormonal effects which have been the object of considerable study (For references, *see* Handler & Orloff, 1973; Sharp & Leaf, 1973; Andreoli & Schafer, 1976).

In addition to the hormonal effects, it is known that a large number of chemical and physical agents affect Na transport; these include polyvalent cations (Martinez-Palomo, Erlij & Bracho, 1971; de Sousa, Marguerat & Grosso, 1973), local anesthetics (Skou & Zerahn, 1959), cholinergic blocking agents (Kirschner, 1955), potential (Mandel & Curran, 1973), hyperosmolarity (Ussing, 1965), and several other agents (for references, *see* Erlij & Ussing, 1978). Instead of listing them individually in the table, we are suggesting that a relatively small number of specific mechanisms underly the actions of many of such apparently unrelated agents. More specifically, we are proposing that many of these agents modify Na transport by interacting with surface charges on the apical side of the epithelium or by modifying the intracellular ion composition of the epithelium. In the following sections we will present some of the observations and arguments which have led us to suggest that modifications in potential and intracellular ion composition may be the general mechanisms involved in the modifications of Na transport caused by many experimental variations.

Materials and Methods

Experiments were made on skins of frogs (*Rana pipiens*). In the cases where the isolated epithelium of the skin was used, it was prepared by the combined use of collagenase and hydrostatic pressure according to a technique described previously (Aceves & Erlij, 1971). Prior to the incubation with the enzyme the *tela subcutanea* was removed under

the dissecting microscope; this minor modification of the original procedure results in the isolation of large areas of epithelium from a single skin.

The measurements of transepithelial potential, short circuit current, and transepithelial fluxes were carried out in skins mounted in chambers similar to those described by Ussing and Zerahn (1951) which were modified to reduce the effects of edge damage (Erlij, 1976).

Total Ca uptake was measured by immersing epithelial pieces (1.54 cm^2) in ^{45}Ca-containing (10 μCi/ml) ^{3}H-mannitol (0.5 Ci/mol) to the incubation medium; unlabeled mannitol was added to provide a final mannitol concentration of 4 mM. At the end of the incubation period the epithelial pieces were digested in 0.2 ml Nuclear Chicago Solvent (NCS) tissue solubilizer and then counted in a toluene mixture in a Packard Scintillation Spectrometer. Quenching was corrected by the External Standards ratio method. Calcium uptake through the apical surface, was measured in skins mounted in Ussing-Zerahn chambers. Radiolabeled Ca together with ^{3}H mannitol were added for one hr to the apical solution only. In all the uptake experiments the solutions contained 5×10^{-5} amiloride.

For efflux measurements the isolated epithelia were first loaded for 1 hr in a solution containing ^{45}Ca; then they were transferred to chambers filled with nonradioactive Ringer's and stirred vigorously. At given intervals the solution was removed from the chamber and replaced by fresh nonradioactive Ringer's. The aqueous samples were counted in Bray's scintillation fluid. At the end of the experiment the epithelial pieces were digested and counted as described above.

Ringer's solution contained (in mmoles/liter): NaCl, 115; KCl, 2.5; $CaCl_2$, 2.0; $MgSO_4$, 1.2; dextrose, 5.0; Tris-HCl, 3.0 (pH 7.4). In most of the experiments in which total ion composition of the outside surface was varied, NaCl was kept constant at 10 mM; buffering was provided by 2 mM Tris Cl, pH 7.4, and the osmolarity ranged between 265 and 268 as measured by an osmometer (Osmette II). To maintain this osmolarity it was necessary to add 220 mmoles of either sucrose or mannitol which were substituted by 130 mmoles of choline when it was desired to increase the total ionic content.

All the experiments were carried at 19 to 22 °C.

Results

On the Role of Surface Potential

Preliminary. One of the more interesting properties of the transport system across tight epithelia is that the rate of Na movement appears to be controlled by the potential difference across the outer border of the epidermis. Already in 1951, Ussing and Zerahn observed that the rate of net Na transport increased as the potential across the skin was reduced. More recently the effects of potential on active transport have been explored in detail (Mandel & Curran, 1973). As the potential across the skin was decreased, active Na transport increased until it reached a saturating level. When the effects of potential were tested at several outside concentrations of Na, they found that different saturation levels are reached at different concentrations. This finding does not conform

with predictions of the effects of potential on a system in which Na would move into the cells by simple diffusion; however, the behavior would fit a system with a voltage-sensitive carrier (Mandel & Curran, 1973). The data did not allow the authors to identify the localization of the voltage-sensitive step of the transport process. However, some recent observations (Biber & Saunders, 1973) show that changes of transepithelial potential markedly modify the uptake of sodium from the outside solution, J_{12}, indicating that the voltage dependence of the transepithelial transport process may be localized in the outer border.

Before the conclusions of Mandel and Curran (1973) are completely accepted, it is necessary to point out that they used urea as a marker of the magnitude of the shunt pathway; since more recently it has been recognized that most of the urea fluxes in tissues in which no precautions are taken to avoid edge damage may occur through the damaged area. Although such a fact may clearly result in erroneous measurements, there is still a possibility that the experiments of Mandel and Curran (1973) provide a first approximation to the true behavior of the tissue if urea was a useful correction of flows through the damaged area. Evidence indicating an important effect of potential in the transport system is also provided by a totally different type of experiment. Cuthbert and Shum (1976) found that depolarization of the skin increases the number of amiloride-binding sites in the skin. This finding is in line with the available electrical and flux measurements and hints that the number of transport sites may be regulated by the potential difference.

In 1957 Huxley (*see* Frankenheuser & Hodgkin, 1957) pointed out that the electric field sensed by a molecule within the cell membrane has a dual origin: one due to transmembrane potential differences, the other originated by the surface potentials set up by fixed charges on the membrane. A corollary of this theory is that the activity of any potential-dependent membrane component ought to be modified not only by changes in external potential but also by alterations of the surface potential. This theory has been more extensively applied in developing an exploration of the shifts in nerve excitability caused by the addition of polyvalent ions (*see* Hille *et al.*, 1975). If a potential-sensitive component is indeed present at the outer border of the skin, changes in the surface potential at this site ought to displace the rate of Na transport along the potential-dependence relationship. If we assume that the outside border of the skin is a negatively charged surface, then according to the Gouy-Chapman theory of surface potential, an increase in the total monovalent concentration, particularly in the absence of

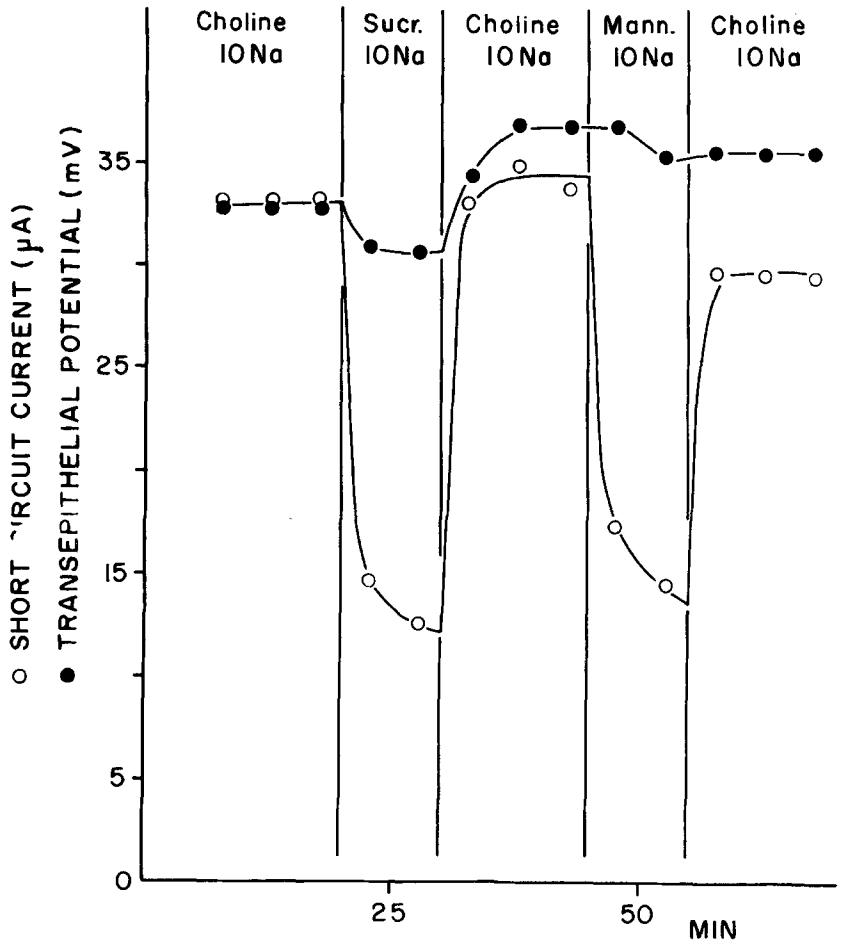

Fig. 1. Effects of reducing total cation concentration of the apical solution on the short-circuit current and potential differences of the frog skin. The potential difference was determined by interrupting the passage of current for 10 sec

divalent or polyvalent cations, should reduce markedly the surface potential. Since the outside of the skin is negative with respect to the inside, the screening of the negative charges on the surface facing the apical solution should have effects analogous to those of depolarizing the skin with electrical currents, i.e., ought to increase transport.

Observations. We have initiated our study by exploring the effects of varying the total ionic concentration on the solution bathing the outside surface of the frog skin. Figure 1 illustrates one of the experiments carried out to measure the effects of modifying the total monovalent

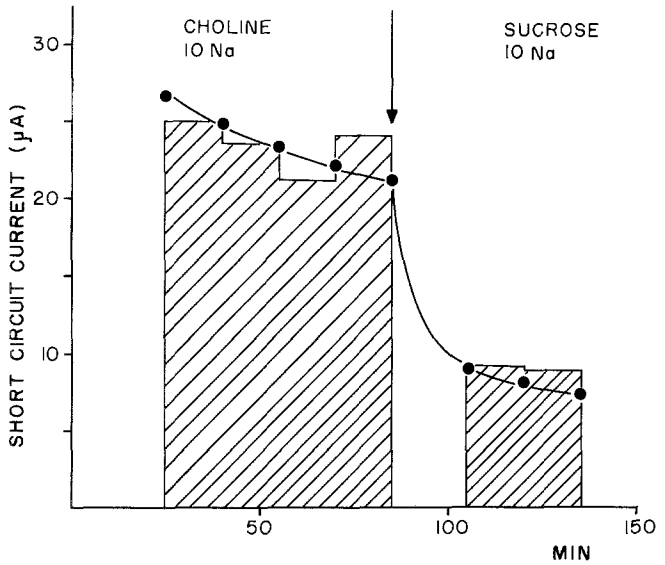

Fig. 2. Relationship between the inward ^{22}Na movement (hatched bars) and the short-circuit current before and after changing total ion content of the frog skin

ion concentration of the outside solution. The Na concentration was kept constant throughout the experiment at 10 mM Ca; and Mg were not included, and the osmolarity of the outside solution was kept constant at 265 mosmoles while total ionic strength was modified by substituting isosmolar amounts of choline chloride by either sucrose or mannitol. The reduction of the total ionic concentration by substituting choline chloride by either sucrose or mannitol caused a large reduction in the short-circuit current across the tissue. Similar results were observed when high ionic concentration was maintained with TEACl. Since we are using the short-circuit current as a measurement of transepithelial Na transport under circumstances in which asymmetric solutions are used, this parameter may not be measuring the transepithelial transport of Na. To check this point we have measured simultaneously the short-circuit current and the transepithelial transport of Na with radiolabeled Na under the conditions of our experiments. One of the experiments in which Na influx, measured with ^{22}Na, and short-circuit current were compared in the same piece of skin is shown in Fig. 2. We have found that the total flux of Na and the short-circuit current coincide under our experimental conditions and that the fall in short-circuit current observed during the reduction of ion content of the outside solution is due to a reduction in the inward movement of Na across the skin. The ratio

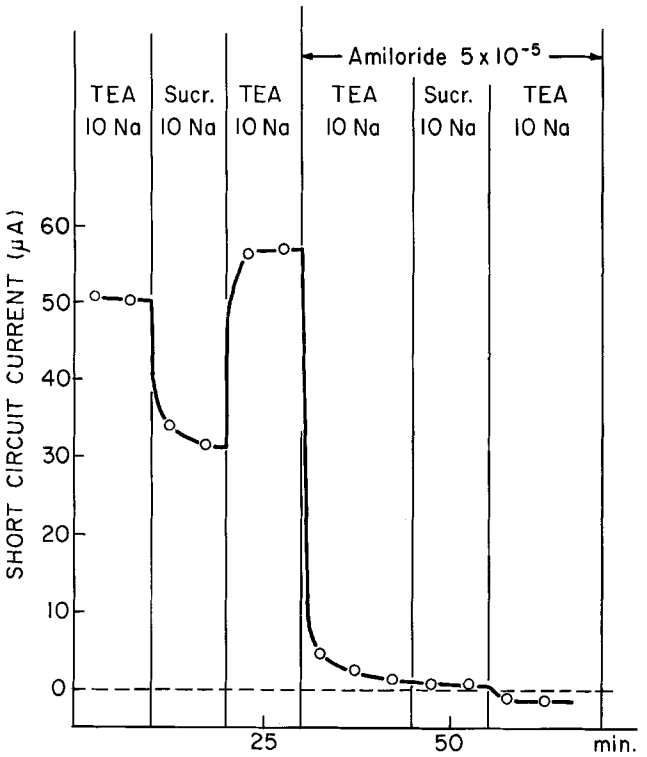

Fig. 3. The effects of amiloride (5×10^{-5}) on the short-circuit current recorded during a change of total ionic content on the apical solution

of short-circuit current over net Na flux expressed as a current (SCC/*I*Na) had an average value of 0.93 ± 0.07 in solutions with high ionic content and of 0.98 ± 0.05 ($n = 5$) in solutions of low ionic content.

There is another observation that indicates that the changes in short-circuit current observed during variations of the ionic content of the solution are not due to a change of ion flows through pathways parallel to the Na transport channel of the skin. When amiloride (10^{-5} M) is added to the outside solution, the entry of Na into the skin is blocked; if then the ion content of the solutions is modified in the presence of amiloride, these changes are without effects on the short-circuit current (*see* Fig. 3).

With respect to the action of divalent and polyvalent cations, it has been known for some time that the addition of La^{+++} and other polyvalent cations to the outside surface of the frog skin produces an increase in the short-circuit current (Martinez-Palomo, Erlij & Bracho, 1971; de Sousa *et al.*, 1973). As shown in Fig. 4, the increase in short-circuit

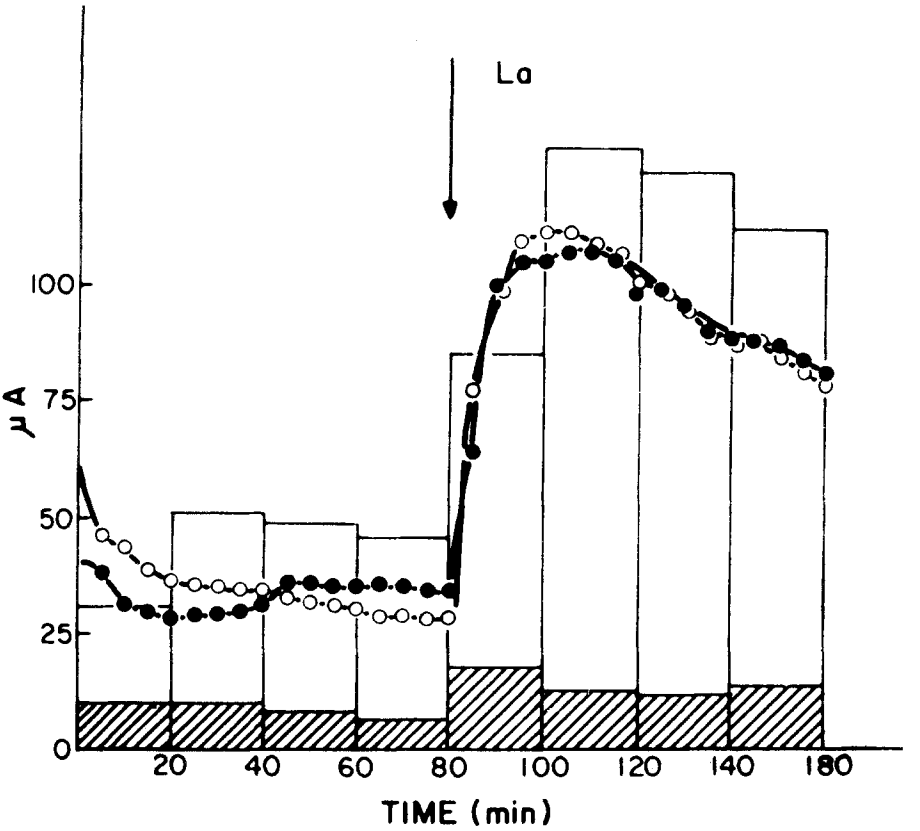

Fig. 4. Relationship between ^{22}Na fluxes measured in paired skins before and after the addition of lanthanum (0.5 mM). The influx (empty bars) was measured in the skin whose current is represented by the empty circles. The efflux (hatched bars) was measured in the skin whose current is represented by the filled circles

current corresponds to an increase in Na transport. Moreover, we have found that the stimulation of short-circuit current caused by lanthanum as well as the resting current are abolished when 5×10^{-5} M amiloride is added to the outside surface of the skin.

The effects of polyvalent cations can be observed with a number of ions. Bracho (1970) in our laboratory determined the effectivity sequence for a number of cations and found it to be $La^{+++} > Ni^{++} > Cd^{++} > Co^{++}$. A stimulatory effect of polyvalent cations, when added to the apical solution of the skin or toad bladder, has now been observed in a number of laboratories (de Sousa *et al.*, 1973; Bentley, Yorio & Fleisher, 1975; Hillyard & Gonick, 1976).

According to the Gouy-Chapman theory, divalent or polyvalent cations are much more effective to screen surface charges than monovalent

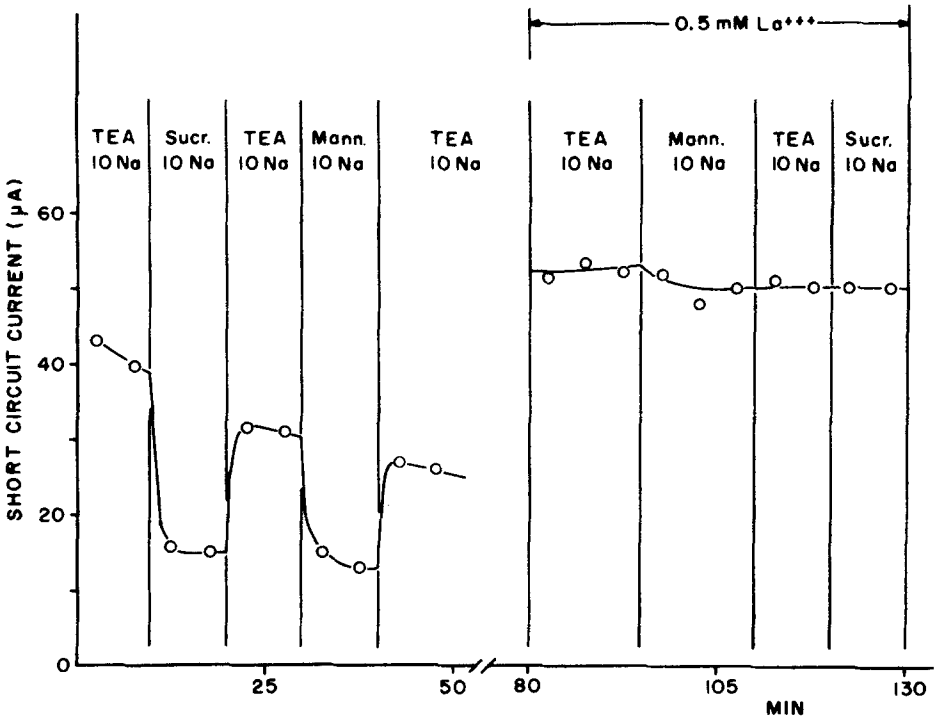

Fig. 5. Modification by lanthanum of the effects of changing total cation concentration on the apical solution. In the first part of the experiment the effects of changing total cation concentration were tested by replacing all the tetraethylammonium by isosmotic amounts of either sucrose or mannitol. Then 0.5 mM La^{++} was added to the outside solution and the effects of reducing cation concentrations were tested again

cations. Therefore, it ought to be expected that the presence of relatively small amounts of polyvalent cations should markedly reduce or abolish the effects of changing monovalent ionic concentration. Figure 5 illustrates one of the experiments carried out to explore this possibility. In this experiment, total monovalent cation concentration was reduced with a constant 10 mM outside Na; TEA was first replaced with sucrose and then with mannitol. The reduction of the total monovalent ion concentration lowered the short circuit current to about 50% of its control value. Afterwards, 0.5 mM La^{+++} was added to the outside solution. This addition increased short-circuit current from 30 to 50 μA/cm^2; then the total monovalent ionic concentration was varied again. When total ionic concentration was modified in the presence of La^{+++} the reduction of the short-circuit current was virtually absent.

Figure 6 summarizes the results of 5 experiments carried out at a constant 10 mM outside Na in which outside ion concentration was varied

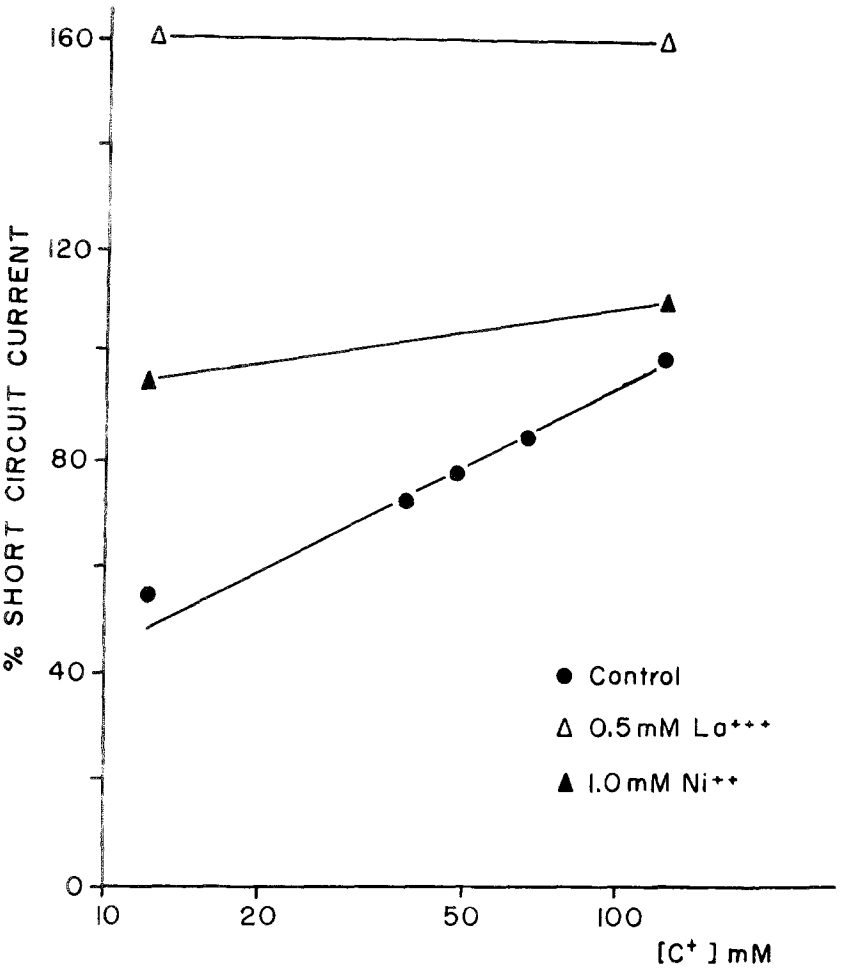

Fig. 6. Comparison of the effects of changing total cation concentration in control preparations (filled circles) and in preparation in which either 0.5 mM La^{+++} (empty triangles) or 1 mM Ni^{++} (filled triangles) has been included in the loading solutions. *Abscissa:* total monovalent cation concentration in the apical solution. *Ordinate:* % change in the short circuit current

in the presence and absence of either Ni^{++} or La^{+++}. In the absence of polyvalent cations, reduction of total monovalent cation concentration reduced the short-circuit current by an average of 56%, while in the presence of polyvalent cations the changes in monovalent cation composition were virtually without effects.

Ionizable acid groups are among the most likely chemical groups that will provide a biological membrane with negative surface charge. In such a case, lowering of pH will reduce the number of dissociated acid groups, and the surface potential will become less negative. For

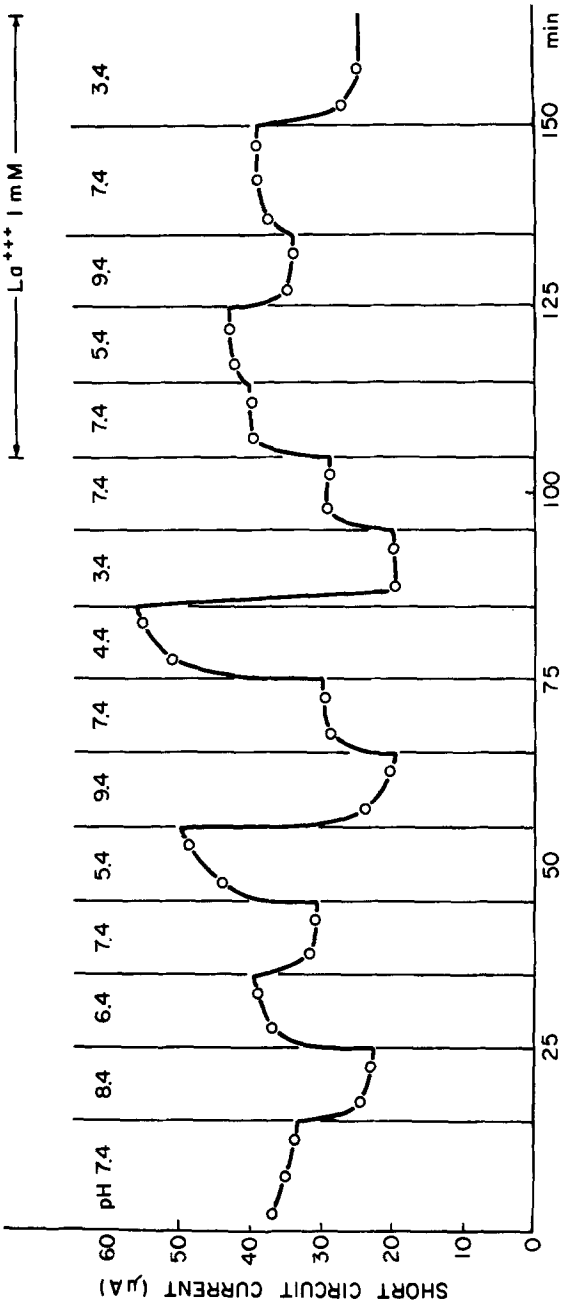

Fig. 7. The effects of successive changes of the outside solution with varying pH values before and after adding La^{+++} (1 mM)

this reason we examined the effects of changing pH of the outside solution on the short-circuit current of skins whose outside surface was bathed in a solution of 10 mM Na and low ionic content. We also explored the effects of La^{+++} on the action of pH on the outside surface of the skin.

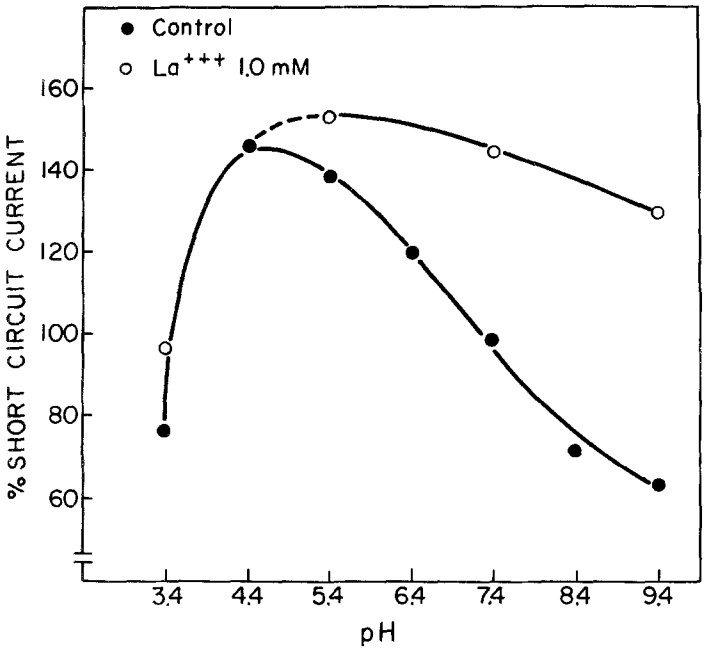

Fig. 8. Effects of varying pH in the presence and absence of lanthanum on the short-circuit current of the frog skin. *Abscissa:* pH. *Ordinate:* % change in the short-circuit current

Figure 7 illustrates a typical experiment. The skin was equilibrated for successive periods in solutions whose pH was varied between 3.4 and 9.4. The current increased as pH was reduced to a value below 4.4. At lower pH values the current was depressed. The effects of modifying pH between values of 9.4 markedly dampened when La^{+++} (1.0 m) was also present in the outside solution. A summary of 4 such experiments is shown in Fig. 8. The short-circuit current recorded at any value of pH was eliminated when amiloride was added to the outside solution. The effects of pH on short-circuit current in the range 4.4 to 9.4 differ from the previous findings of Schoffeniels (1956) and are similar to more recent observations in the toad bladder (Leaf, Keller & Dempsey, 1964) and skin (Lindemann & Voûte, 1976).

The curve suggests that there are two types of effects of varying pH. The effects observed at pH values above 4.4 are compatible with the possibility that H^+ ions reduce the negative charge of the outer border of the skin. The effects at pH values below 4.4 may represent an interaction at a second group of sites that results in a blockade of the Na channel as suggested by Lindemann and Voûte (1976). One of the difficulties in interpreting experiments in which ion substitutions

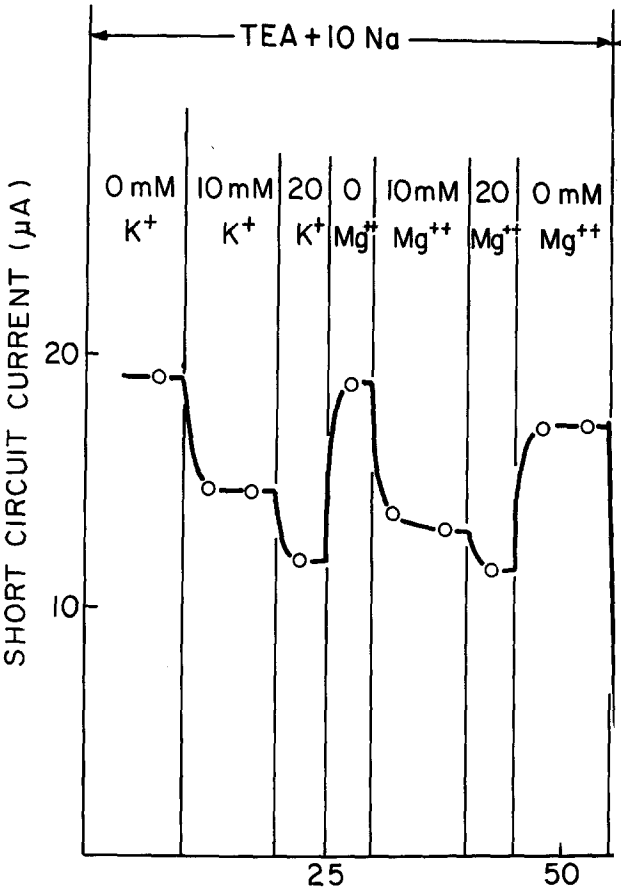

Fig. 9. The effects of adding K^+ or Mg^{++} to the apical solution on the short-circuit current of the frog skin

are performed is to identify "neutral" cations for changing the ionic strength. For example, there is some evidence that K^+ does not act simply as an inert ion that is not admitted in the Na pathway of the skin, but that it also blocks the movement of Na across the outer border of the skin (Rotunno *et al.*, 1970). The experiment in Fig. 9 provides additional evidence in agreement with this view. The experiment was carried out with a constant 10 mM Na throughout. The short-circuit current was first determined in a solution in which concentration in the medium was increased by substituting 10 mM K for an isosmolar amount of TEA. The short-circuit current was depressed by the addition of K^+. A further increase of K^+ caused an additional reduction of the short-circuit current.

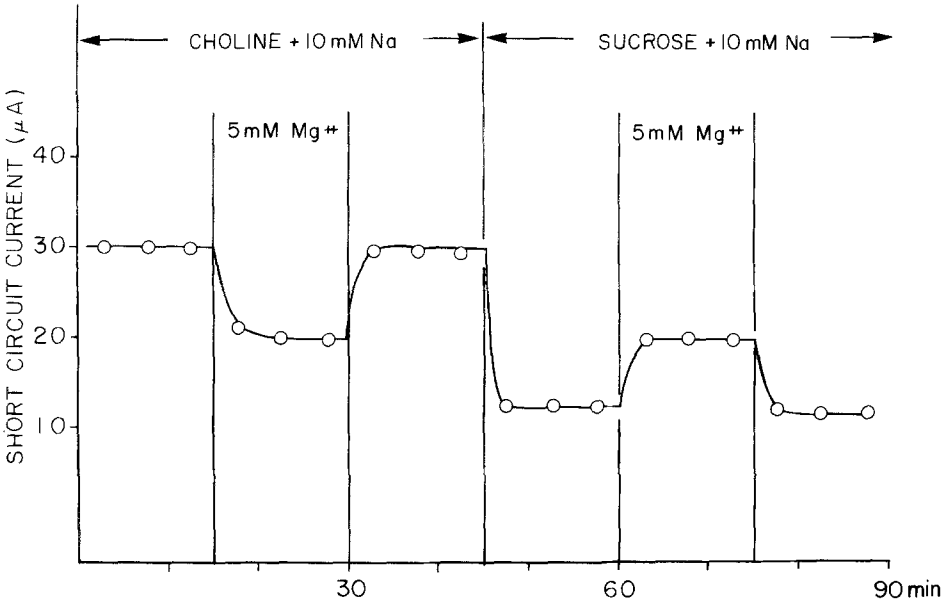

Fig. 10. Effects of increasing Mg concentration on a skin that first had a high ionic content solution on its outside surface. The same procedure was repeated when the outside solution had low ionic content

In the following part of the experiment it is shown that the addition of Mg^{++} has similar effects to K^+. After replacing the outside solution with the initial solution, the composition of the solution was altered by substituting TEACl by $MgCl_2$. This replacement again produced a reduction of the short-circuit current. An inhibition of Na^+ entry into the skin by alkali earth cations has been also reported earlier (Curran, Herrera & Flanigan, 1963), and it will be further considered below. In the following part of the experiment all the TEA was substituted for K^+ and then the effects of gradually substituting TEA for K^+ were tested. The addition of TEA had no stimulatory effects. Similar inhibitory effects of K^+ and Mg^{++} were also observed when choline chloride was used to keep the osmolarity of the medium constant.

The inhibitory effects of some cations on the entry process may provide some explanation for one of the difficulties of the surface potential hypothesis: the fact that the published experiments on the effects of alkali earth cations show that these agents decrease Na transport instead of decreasing it as predicted by theory. If alkali earth cations have dual effects: one at the surface charges and the other at the Na channel itself, it may be possible to separate them by selecting appropriate

experimental conditions. A start in that direction is shown in Fig. 10. In this case we have compared the effects of the addition of 2 mM Mg^{++} on the short-circuit current of skins exposed to solutions of low ionic content at the outer surface with the effects observed in solutions of elevated ion content. The addition of Mg has opposite effects in both conditions. In the case of low ionic content it increases Na transport while in solutions of high ionic content it reduces short circuit current. Again this experiment can be interpreted in line with the surface charge hypothesis; under low ionic content conditions, the effect of Mg in screening surface charge predominates over other effects that this ion may have on the transport system, while in the high ionic-content solutions, where a greater fraction of the surface charge is screened, the predominant effect of Mg is an inhibition of transport.

The Effects of Intracellular Ca

We became interested in the role of intracellular Ca in the regulation of transepithelial Na transport while trying to find an explanation for the observation that elimination of Na from the basal surface of the skin causes a drastic reduction in transepithelial Na transport (Mandel & Curran, 1973; Rabito, Rodriguez-Boulan & Cereijido, 1973). Since one of the more general effects of Na-free solutions is to increase intracellular Ca levels (Baker, 1972; Blaustein, 1974), we decided to explore the effects of Na-free solutions on the fluxes of ^{45}Ca across the cell membranes of the isolated epithelium of the frog skin.

The main findings of our studies (Erlij & Grinstein, 1977; Grinstein & Erlij, 1978) can be summarized as follows:

1) Sodium-free solutions cause a threefold stimulation of ^{45}Ca uptake; nearly all the increased uptake occurs through the basal border.

2) Sodium-free solutions reduce ^{45}Ca efflux from the cells by about 50%.

3) The reduction in transepithelial Na transport caused by Na-free solutions is abolished when Ca^{++} is also eliminated from the Na-free solutions.

The experiments indicate that Na-free solutions increase the levels of Ca in the epithelial cells since they increase Ca influx and decrease Ca efflux. Moreover, they suggest that the increase of cell Ca is associated

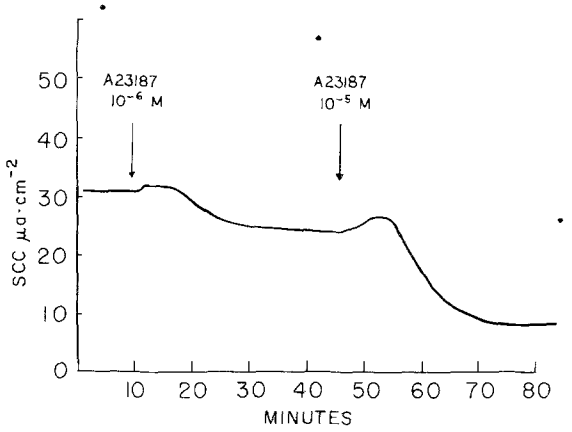

Fig. 11. Effects of ionophore A23187 on the short-circuit current of the isolated epithelium of the frog skin. The dots mark the level of potential reached when current passage was interrupted for 10 sec. The scale of current is in $\mu A/cm^2$, and mV for the voltage readings

with the reduction of Na transport since preventing the entry of calcium into the cells abolishes the inhibition of transport.

In more recent experiments we have pursued further the role of intracellular Ca by examining the effects of the ionophore A23187 on the transepithelial transport of Na in the isolated epithelium. This agent selectively increases membrane permeability to divalent cations (Reed, 1972; Case, Vanderkooi & Scarpa, 1974). Figure 11 shows the effects of the ionophore on the short-circuit current of an isolated epithelium. The ionophore was added to the basal solution after a stable base-line period had been established. The addition of 10^{-6} M of A23187 produced a small reduction of transport; when 10^{-5} M were added an initial and short lasting increase in short-circuit current was produced followed by a decline in short-circuit current. In 8 similar experiments the ionophore reduced short-circuit current to 30% of its original level. In other experiments we have corrobrated with determinations of ^{22}Na fluxes that the fall in short-circuit current is due to a reduction of Na transport.

These findings with the Ca ionophore provide further support for the notion that increased intracellular Ca inhibits transepithelial Na transport. A recent communication shows that the ionophore has similar effects on Na transport in the toad urinary bladder (Weismann, Sinha & Klahr, 1977) and suggests that cell Ca may also inhibit Na transport in the amphibian urinary bladder.

Discussion

The results described in this communication are in agreement with the notion that both changes in surface potential and cell Ca levels can act as regulatory mechanisms of transepithelial Na transport. The changes in Na transport caused by variations in total ionic content, pH, and polyvalent cations are consistent with the suggestion that their effects are due to a modification in the negative surface potential in the apical border of the skin. Indeed, increases in the concentrations of monovalent cations, H^+, and polyvalent cations have effects analogous to those of electrical depolarization of the frog skin.

Among the more intriguing observations in the literature that can be interpreted along the lines of interactions with surface charges are the experiments of Skou & Zerahn (1959) on the effects of local anesthetics in the frog skin. The anesthetics stimulated the transport of sodium when added to the outside solution in the ionized form, while the undissociated molecules were without effects. Other cationic molecules stimulate Na^+ transport when added to the outside solution; these include atropine and curare (Kirschner, 1955) and some amiloride derivatives (Zeiske & Lindemann, 1974); their effect could also be mediated through screening of surface charges.

It is also tempting to speculate that the effects of changing osmolarity of the inside solution (Ussing, 1965) are mediated by modifications of the surface potential. Thus, reduced osmolarity swells the cells and reduces total ionic concentration in their interior. This effect would tend to increase the negative surface potential at the intracellular surface of the membrane that faces the intracellular medium and ought to have effects equivalent to a depolarization. Indeed, dilution of the inside solution increases transport, whereas hyperosmotic solutions decrease transport (Ussing, 1965).

There is a possibility that we have not ruled out, i.e., that all the modifications we have described are not due to a change in surface potential but to the interaction of cations with a "modifier" site. If such a "modifier" site is responsible for the response to such a large variety of agents, it must have very low selectivity; although possible, we consider it unlikely that the transport system would possess a potent modifier site that can combine with almost any positive substance present in the bathing solution.

The experiments on the effects of procedures that increase cell Ca suggest that the level of this ion within the cell may play an important

role in regulating transepithelial Na transport. There are at least two modes in which increased Ca levels could inhibit transepithelial transport: either by decreasing the permeability of the apical membranes to Na or by inhibiting the activity of the NaK-dependent ATPase on the basolateral surface of the skin. The inhibition caused by reduced serosal Na or the antibiotic is associated with an increased transepithelial resistance. Since this parameter is essentially determined by the Na permeability of the apical membrane (Higgins *et al.*, 1975; Erlij, 1976; Lewis & Diamond, 1976), it is possible that the Na permeability of the apical border is reduced by increased intracellular Ca^{++}. However, the possibility that increased cell Ca^{++} also acts at the basolateral border cannot be excluded for the time being. Thus, it is well known that increased cell Ca^{++} inhibits the Na-K pump (Bodemann & Hoffman, 1976), while in the frog skin K^{+} uptake by the basolateral surface is inhibited in Na-free solutions (Curran & Cereijido, 1965).

Of the agents listed in Table 1 we have not discussed the role of cell Na, since the evidence for its possible role in the regulation of transport has already been discussed in other publications (Erlij & Smith, 1973; Leblanc & Morel, 1975; Ussing, Erlij & Lassen, 1974).

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