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Apical Na^+ Permeability of Frog Skin during Serosal Cl^- Replacement

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Summary. Gluconate substitution for serosal Cl^- reduces the transepithelial short-circuit current (I_{sc}) and depolarizes short-circuited frog skins. These effects could result either from inhibition of basolateral K^+ conductance, or from two actions to inhibit both apical Na^+ permeability (P_{Na}^{ap}) and basolateral pump activity. We have addressed this question by studying whole- and split-thickness frog skins. Intracellular Na^+ concentration (c_{Na}^c) and P_{Na}^{ap} have been monitored by measuring the current-voltage relationship for apical Na^+ entry. This analysis was conducted by applying trains of voltage pulses, with pulse durations of 16 to 32 msec. Estimates of P_{Na}^{ap} and c_{Na}^c were not detectably dependent on pulse duration over the range 16 to 80 msec. Serosal Cl^- replacement uniformly depolarized short-circuited tissues. The depolarization was associated with inhibition of I_{sc} across each split skin, but only occasionally across the whole-thickness preparations. This difference may reflect the better ionic exchange between the bulk medium and the extracellular fluid in contact with the basolateral membranes, following removal of the underlying dermis in the split-skin preparations. P_{Na}^{ap} was either unchanged or increased, and c_{Na}^c either unchanged or reduced after the anionic replacement. These data are incompatible with the concept that serosal Cl^- replacement inhibits P_{Na}^{ap} and Na,K-pump activity. Gluconate substitution likely reduces cell volume, triggering inhibition of the basolateral K^+ channels, consistent with the data and conclusions of S.A. Lewis, A.G. Butt, M.J. Bowler, J.P. Leader and A.D.C. Macknight (*J. Membrane Biol.* **83**:119–137, 1985) for toad bladder. The resulting depolarization reduces the electrical force favoring apical Na^+ entry. The volume-conductance coupling serves to conserve volume by reducing K^+ solute loss. Its molecular basis remains to be identified.

Key Words short-circuit current · gluconate · intracellular potential · K^+ conductance · Na,K-exchange pump · cell volume

Introduction

Sodium transport across tight epithelia is strongly dependent on the anionic composition of the external media (Singer & Civan, 1971; Civan, 1983). For example, total replacement of external Cl^- by gluconate reduces the short-circuit current (I_{sc}) and mucosal-to-serosal flux of radioactive Na^+ across frog skin, while producing a smaller increase in K^+ flux in the opposite direction (Nielsen, 1984). Similar effects can be elicited by replacing Cl^- solely in the serosal medium bathing the isolated epithelium: the short-circuit current and transepithelial conductance are thereby reduced (Duffey et al., 1986).

The ionic basis for the Cl^--dependent reduction in transepithelial current is unknown. External Cl^- replacement has been reported to produce intracellular depolarization of the short-circuited skin, which may be preceded by a transient hyperpolarization of variable magnitude (Biber et al., 1985; Duffey et al., 1986). The observed reductions in short-circuit current, transepithelial conductance, and apical membrane potential cannot result from a single action to reduce apical Na^+ permeability (P_{Na}^{ap}). However, the observations are consistent with either: (i) a reduction in the basolateral K^+ permeability [as suggested by the observations of Lewis et al. (1985) in toad urinary bladder], or (ii) two separate actions of Cl^- replacement, to reduce apical Na^+ permeability and to inhibit the basolateral Na,K-exchange pump (Duffey et al., 1986).

Regulation of apical Na^+ permeability has been considered to play a major role in modulating transepithelial Na^+ transport (Lichtenstein & Leaf, 1965; Lewis, Eaton & Diamond, 1976; Civan, 1983). Given its general importance and its possible specific role in mediating anion-dependent effects on Na^+ transport, we have measured P_{Na}^{ap} electrophysiologically in the present work. These results indi-

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cate that gluconate substitution for serosal Cl^- neither reduces apical Na^+ permeability nor increases intracellular Na^+ concentration.

Materials and Methods

Transepithelial Measurements

After excision from doubly pithed frogs (*Rana pipiens pipiens;* West Jersey Biological Supply, Wenonah, N.J.), abdominal skins were rinsed and bathed with an aerated Cl^- Ringer's solution (DeLong & Civan, 1984) containing (in mM): 118.2 Na^+, 3.4 K^+, 0.9 Ca^{2+}, 117.2 Cl^-, 2.3 HCO_3^-, 1.8 HPO_4^{2-}, and 0.3 $H_2PO_4^-$, at a pH of 7.5 and an osmolality of 223 to 228 mOsm. During the course of the experimental measurements, the serosal surfaces were perfused with either the Cl^--Ringer's solution or with a gluconate-Ringer's medium free of Cl^- (Civan et al., 1984), which contained: 120.0 Na^+, 3.5 K^+, 3.0 Ca^{2+}, 122.0 gluconate, 2.5 HCO_3^-, and 10.0 HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid), at a pH of 7.5 and an osmolality of 230 mOsm. The higher concentration of Ca^{2+} in the gluconate-Ringer's solution was necessary in order to maintain the Ca^{2+} activity reasonably high; the concentration of free Ca^{2+} in this medium has been measured with Ca^{2+}-selective microelectrodes to be 0.8 mM (Duffey et al., 1986). The Cl^-- and gluconate-Ringer's media were buffered with phosphate and HEPES, respectively; the electrophysiologic properties of frog skin are similar when either buffer is used (Civan et al., 1983). The mucosal surface was usually perfused with a nitrate-Ringer's solution (identical with the Cl^--Ringer's solution except for the equimolar replacement of NO_3^- for Cl^-) in order to minimize any potential changes in paracellular conductance (Nagel, Garcia-Diaz & Essig, 1983).

Full-thickness preparations were mounted mucosal-surface-up between the two halves of a Lucite® chamber (DeLong & Civan, 1978), exposing 1.9 cm^2 of tissue for study. The transepithelial potential (ψ^{ms}, serosal with respect to mucosal solution) was alternately clamped at 0 mV for 7.7 sec (to monitor short-circuit current) and at 10 to 20 mV for 4.3 sec (to monitor transepithelial conductance).

Isolated epithelial preparations were prepared by preincubating the skins with collagenase (Fisher, Erlij & Helman, 1980). These split-thickness preparations were mounted serosal-side-up and impaled across their basolateral membranes.

Intracellular Measurements

Both apical and basolateral impalements were conducted with single-barrelled micropipets drawn from omega-dot capillary glass tubing (1.5 mm outer and 0.75 mm inner diameter; Glass Co. of America, Millville, N.J.). The filling solution contained 0.5 M KCl to reduce the rate of release of saline into the cell during the intracellular recordings. Minimal criteria for acceptability of an intracellular impalement have been presented elsewhere (Civan et al., 1983; DeLong & Civan, 1983). All of the measurements of intracellular potential (ψ^{mc}, cell with respect to mucosal medium) were obtained after ψ^{mc} was stable for at least several minutes. Most of the data were collected during the course of continuous intracellular monitoring for prolonged periods of 30 min or longer. Both intracellular potential and transepithelial current were continuously displayed on a storage oscilloscope and a dual-pen paper chart recorder.

Pulse Trains

The measurement of apical Na^+ permeability and of intracellular concentration of free Na^+ (c^c_{Na}) was based upon the approach of Fuchs, Larsen and Lindemann (1977). A train of transepithelial voltage pulses was applied before and after adding mucosal amiloride (20 to 100 μM), which reversibly blocks the apical Na^+ channels (Benos, 1982). Like Fuchs et al. (1977), we have measured the Na^+ current across the apical plasma membrane (I^{mc}_{Na}) as the amiloride-sensitive transepithelial current, although ψ^{mc} has been measured directly with intracellular micropipets [rather than by measuring the transepithelial potential of the depolarized preparation (Fuchs et al., 1977)]. The measured relationship between (I^{mc}_{Na} and ψ^{mc} is fit by the Goldman equation (Goldman, 1943):

$$I^{mc}_{Na} = -[P^{ap}_{Na}\beta F][(c^m_{Na} - c^c_{Na}e^{\beta})/(1 - e^{\beta})] \quad (1)$$

where $\beta = [\psi^{mc}F/(RT)]$, F is the Faraday constant, R is the perfect gas constant, T is the absolute temperature, and c^m_{Na} is the mucosal Na^+ concentration. P^{ap}_{Na} and c^c_{Na} can be estimated from the best fit of the data since all of the other parameters of Eq. (1) are known.

The pulse trains consisted of 12 pairs of alternately hyperpolarizing and depolarizing pulses, whose magnitudes increased by 20 mV in successive steps. The transepithelial potential was returned to 0 mV after each voltage pulse. In our initial program, the durations of each pulse (Δt_P) and interpulse interval (ΔT_{ip}) were fixed (DeLong & Civan, 1984). In the present work, the program has been modified to permit variation of both time periods over wide ranges. In our earlier study (DeLong & Civan, 1984), (Δt_P) was fixed at 31 msec. In the current work, (Δt_P) was extended to values as large as 80 msec, while (Δt_{ip}) was extended to periods as long as 40 $\times$ Δt_P. Just before terminating each voltage step, ψ^{mc}, ψ^{ms} and the transepithelial current were consecutively sampled eight times over 0.96 msec. The means of the sets of eight data points were used in the subsequent data analysis.

All data are presented as the mean ± SE. Unless otherwise stated, the probability (P) of the null hypothesis has been calculated with Student's t-test.

Chemicals

Amiloride was kindly provided by Dr. George M. Fanelli, Jr. (Merck Institute for Therapeutic Research, West Point, Pa.) and stored as concentrated aqueous solutions at 4°C. Collagenase (CLS II) was obtained from Worthington (Millipore, Freehold, N.J.).

Results

Measurements of P^{ap}_{Na}

In principle, the pulse duration must be carefully selected in measuring P^{ap}_{Na} with the electrophysiologic approach described in Materials and Methods. Step changes in transepithelial voltage or current can result in time-dependent changes in the conju-

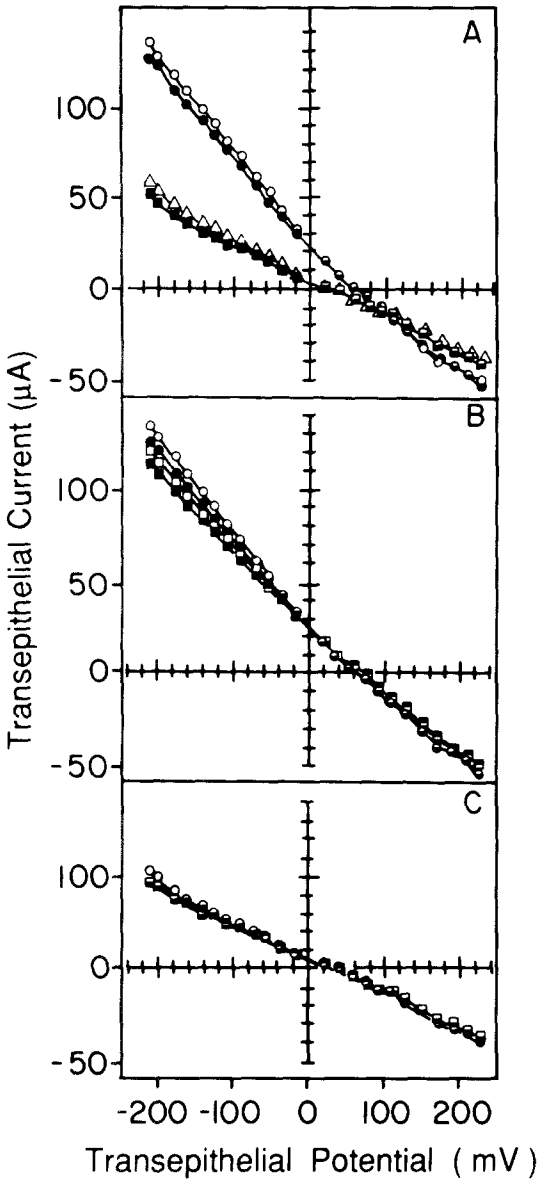

Fig. 1. Transepithelial current-voltage relationship ($I_T - \psi^{ms}$) as a function of duration (Δt_P) of voltage pulses applied across a split-thickness skin. (*A*) All data have been collected with (Δt_P) = 16 msec in order to illustrate the reproducibility of the measurements. The open and filled circles indicate data points obtained under baseline conditions (without amiloride), with an intervening period of 4 min. The open and filled squares and open triangles symbolize data points collected over a 10-min period in the presence of 100 μM mucosal amiloride. In this and the other two panels, the connections between the data points have been introduced as an aid in identifying the sets of results, and have no theoretical significance. (*B*) The results have been obtained during the same 4-min baseline period as that bracketed by the open and closed circular data points of panel (*A*). In panel (*B*), the symbols for the different pulse durations are: open circles, 16 msec; filled circles, 32 msec; open squares, 50 msec; and filled squares, 80 msec. (*C*) The results have been obtained in the presence of 100 μM mucosal amiloride during the same 10-min period as that of the triangular and square data points of panel (*A*). The four sets of data points, represented in panel (*C*) by open and closed circles and squares, were obtained with pulse durations of 16, 32, 50 and 80 msec. With amiloride present, the four sets of $I^{ms} - \psi^{ms}$ relationships are almost completely superimposed

gate parameter, which are not ascribable to simple capacitative responses (Weinstein et al., 1980). At least some of these time-dependent effects likely reflect polarization potentials and impedances resulting from ionic translocations during prolonged stimulation. These polarization effects can be observed over time frames of seconds to minutes. The data of Fig. 1 suggest that similar time-dependent effects may be observed over periods as brief as 50 to 80 msec. Figure 1(*A*) illustrates that measurements of transepithelial current (I_T) as a function of transepithelial potential (ψ^{ms}) are reproducible at different points in time using the same pulse duration (Δt_P = 16 msec). Figure 1(*B*) presents the $I_T - \psi^{ms}$ relationship as a function of Δt_P over the range 16 to 80 msec. The curves are nearly superimposed when $\psi^{ms} > 0$ (serosa positive with respect to mucosa), but display considerable splay with time following strongly depolarizing currents (upper left quadrant). This time-dependent splay is not likely to reflect simple capacitative responses. Otherwise, we would have expected symmetrical time-dependent effects following application of hyperpolarizing currents of similar magnitude and duration, contrary to observation (lower right quadrant). The time dependence illustrated by Fig. 1(*B*) is also unlikely to reflect transient changes induced in the intercellular tight junctions and lateral intercellular spaces (Bindslev, Tormey & Wright, 1974; Finn & Bright, 1978) of frog skin since the splay of the $I_T - \psi^{ms}$ relationship was abolished when the transcellular transepithelial conductance of the same tissue was blocked with amiloride (Fig. 1*C*).

The data of Fig. 1 have suggested to us that noncapacitative effects may be produced by prolonged voltage stimulation, that such effects may appear after only 50 to 80 msec, and that these complexities are likely to be most prominent with strongly depolarizing voltage steps. Although this consideration indicates that Δt_P should be preferably < 50 to 80 msec, Δt_P should ideally also be longer than the period of the capacitative transient. It should be noted that, at least for the capacitative transient response of ψ^{mc} to step changes in transepithelial voltage, the magnitude of the transient depends not only on the magnitude of the apical time constant, but also on the difference between the time constants of the apical and basolateral membranes (DeLong & Civan, 1984). When the two time constants are equal, no transient response will be detected in ψ^{mc}, irrespective of their absolute value.

Table 1. P_{Na}^{ap}, c_{Na}^{c} as functions of pulse duration (Δt_P)[a]

Parameter	Mean value (entire range of Δt_P)	Deviations from mean			
		16 msec	32 msec	50 msec	80 msec
P_{Na}^{ap}	11	0.05	0.03	0.11	−0.4
(10^{-7} cm^3 · sec^{-1})	±4(6)	±0.06(6)	±0.04(6)	±0.08(6)	±0.2(3)
c_{Na}^{c}	10	0.12	0.4	−0.6	−0.8
(mM)	±2(6)	±0.05(6)	±0.3(6)	±0.4(6)	±0.7(3)

[a] A series of six split-thickness frog skins was studied by applying a train of transepithelial voltage pulses of varying duration. The number of skins studied with each pulse duration is indicated in parentheses. The means ± SE have been obtained by averaging the values obtained over the full range of pulse durations. The relatively large uncertainty associated with the mean absolute value of P_{Na}^{ap} reflected biological variation from tissue to tissue, rather than the uncertainty of estimating this parameter in any given preparation. The four columns to the right display the mean ± SE values for the corresponding values measured with each of the four sets of pulse durations. These values are presented as the deviations from the mean; a value of 0 would indicate that the measured value was identical with the mean for the entire range of pulse durations applied to the skin studied.

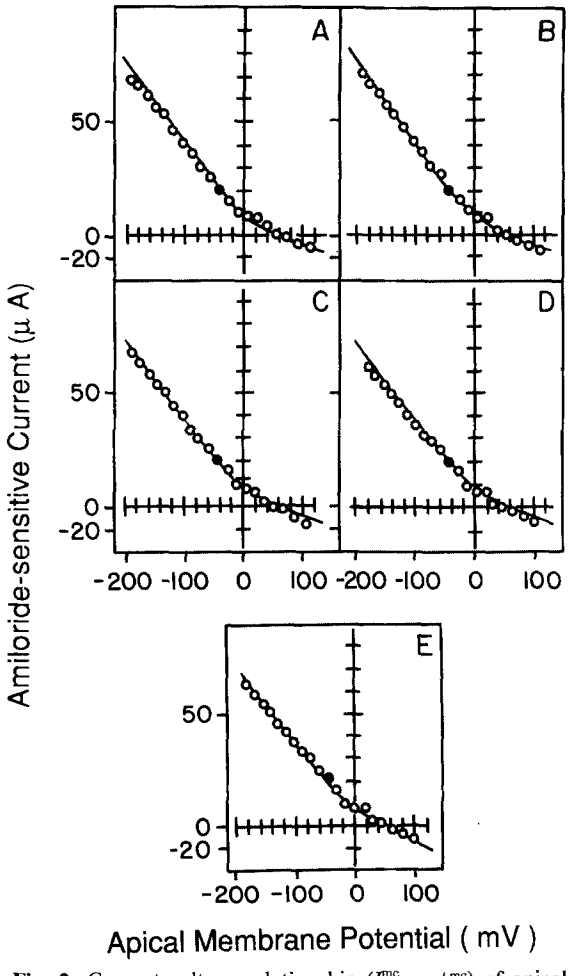

Fig. 2. Current-voltage relationship ($I_{Na}^{mc} - \psi^{mc}$) of apical Na^+ entry as a function of pulse duration. The results have been calculated from four consecutive trains of pulses applied during a single intracellular penetration of a split-thickness preparation. The pulse durations used were: (*A*) 16 msec, (*B*) 32 msec, (*C*) 50 msec, and (*D–E*) 80 msec. The solid lines have been generated with the Goldman equation, setting c_{Na}^{c} = 16.8 mM and P_{Na}^{ap} equal to either 8.4 × 10^{-7} (panels *A–D*) or 8.1 × 10^{-7} cm^3 · sec^{-1} (panel *E*)

Thus, the electrophysiologic data can be usefully analyzed even before the capacitative response has been fully dissipated, so long as the capacitative component is small with respect to the contribution from the conductive channels.

In order to choose an optimal pulse duration for studying P_{Na}^{ap} and c_{Na}^{c}, we have measured these parameters as a function of Δt_P in the same tissues. The results of our previous study (DeLong & Civan, 1984), indicated that the estimates of P_{Na}^{ap} and c_{Na}^{c} were not strongly dependent upon pulse duration over a range of values of $\Delta t_P < 31$ msec. In the present work, we have examined the effects of increasing Δt_P beyond that range. Figure 2 presents data obtained during a single continuous impalement, in which Δt_P was fixed at 16 (*A*), 32 (*B*), 50 (*C*) and 80 msec (*D*, *E*) in applying successive trains of voltage pulses. The solid lines have been constructed from Eq. (1), taking c_{Na}^{c} to be 16.8 mM. All of the data sets can be reasonably well fit by fixing P_{Na}^{ap} = 8.4 × 10^{-7} cm^3 · sec^{-1} (panels *A–D*); the fit is minimally improved for the data collected with 80-msec pulses when P_{Na}^{ap} is taken to be slightly smaller (8.1 × 10^{-7} cm^3 · sec^{-1}, panel *E*). Thus, the data of Fig. 2 suggest that the estimated values of P_{Na}^{ap} and c_{Na}^{c} are little changed when Δt_P is varied over the range 16 to 80 msec.

This conclusion is further supported by the results of Table 1. In a series of six skins, P_{Na}^{ap} and c_{Na}^{c} were estimated from analysis of pulse trains conducted with varying pulse durations. For each skin, the mean value for each parameter was calculated, and the deviations from the means were obtained as a function of pulse duration. After the results from the six preparations are averaged (Table 1), no significant deviations from the mean values are noted at any of the pulse durations used. We conclude from Fig. 2 and Table 1 that the electro-

Table 2. Effects of serosal Cl^- replacement by gluconate[a]

Side impaled	Baseline measurements				Changes after Cl^- replacement			
	ψ_{sc}^{mc} (mV)	I_{sc} ($\mu A \cdot cm^{-2}$)	P_{Na}^{ap} (10^{-7} $cm^3 \cdot sec^{-1}$)	c_{Na}^{c} (mM)	ψ_{sc}^{mc} (mV)	I_{sc} ($\mu A \cdot cm^{-2}$)	P_{Na}^{ap} (10^{-7} $cm^3 \cdot sec^{-1}$)	c_{Na}^{c} (mM)
Basolat.	−69	18	8.3	12.3	37[e]	−6.4[c]	0.2	−6.9[d]
	±3	±3	±1.5	±0.6	±4	±1.6	±0.7	±1.2
	(5)	(5)	(5)	(5)	(5)	(5)	(4)	(4)
Apical	−70	30	17	21	21[f]	1	6*	−4
	±3	±4	±3	±3	±3	±2	±2	±3
	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)

[a] The values entered are the means ± SE, with the number of experiments in parentheses. The entries under ψ_{sc}^{mc} are the measurements of apical membrane potential in the short-circuited state ($\psi^{ms} = 0$). The symbol * indicates that the probability of the null hypothesis was <0.02 calculated by the nonparametric coin test. The other superscripts are the probabilities of the null hypothesis calculated on the basis of Student's t-test: (b) <0.05, (c) <0.02, (d) <0.01, (e) < 0.005, and (f) <0.001.

physiologic estimation of P_{Na}^{ap} and c_{Na}^{c} is not critically dependent on the choice of Δt_P over the range 16 to 80 msec. In the second part of this study (analysis of the effects of serosal Cl^- replacement), Δt_P was taken to be either 16 or 32 msec. The interpulse interval has not been found to be a critical parameter, as long as a sufficiently long interval is used to permit the membranes to relax after each pulse. Values of either $15 \times \Delta t_P$ or $40 \times \Delta t_P$ were used in the second half of the work.

In our previous study, and in the first phase of the current research, the choice of satisfactory Goldman fits was conducted by inspection. In order to avoid possible bias in the processing of the data, the best fits were chosen by a nonlinear least-squares analysis in the second phase of the study. Similar conclusions were reached by application of either technique of data reduction.

Basolateral Impalements

Frog skin epithelium can be impaled from either surface. In our hands, it is somewhat easier to obtain stable intracellular recordings by penetrating the cell from the mucosal surface. Such apical penetrations are performed with the whole-skin preparation, precluding the need for enzymatic treatment and possible modification of the transport characteristics. On the other hand, impalement-induced shunting is less likely to alter the intracellular potential measured with impalements from the serosal medium, because of the higher conductance of the basolateral membrane (Higgins, Gebler & Frömter, 1977). Furthermore, the enzymatic removal of the underlying dermis improves ionic exchange between the bulk serosal medium and the extracellular medium in contact with the basolateral membranes (Fisher et al., 1980). In the present study, intracellular penetrations have been performed across both plasma membranes.

As previously reported (Duffey et al., 1986), gluconate replacement of serosal Cl^- promptly and uniformly reduced the short-circuit current across split-thickness skins. The depression of current was monotonically progressive over periods of minutes. In addition, the basolateral impalements also confirmed our previous observation (Duffey et al., 1986) that the serosal Cl^- replacement produced a progressive apical depolarization of the short-circuited isolated epithelium. The time course of the membrane depolarization was similar to that of the inhibition of short-circuit current. Averaging the results for a series of four experiments (Table 2), the mean depolarization was 37 ± 4 mV.

Measurements of P_{Na}^{ap} indicated that the anionic substitution was unassociated with any significant change in apical Na^+ permeability in this series of experiments (Table 2). On the other hand, the fall in I_{sc} was accompanied by a significant reduction in intracellular Na^+ concentration of 7 ± 1 mM (Table 2).

The data obtained with the isolated epithelia suggest that the Cl^- substitution triggered no direct effect on P_{Na}^{ap}, but reduced the rate of Na^+ entry by decreasing the electrical driving force (ψ^{mc}). With the continued activity of the basolateral Na,K-exchange pump, c_{Na}^{c} fell to a lower steady-state level. The reduced availability of Na^+ substrate then led to a lower rate of Na^+ extrusion by the pump.

Apical Impalements

In contrast to the transepithelial measurements of the isolated epithelia, gluconate substitution for serosal Cl^- was not usually associated with striking changes in current and conductance (g_T) across the

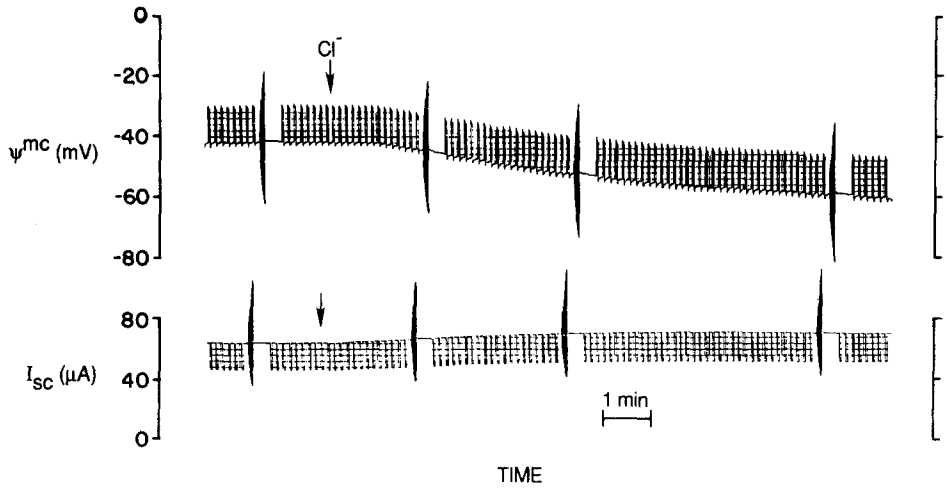

Fig. 3. Effects of restoring serosal Cl^- on intracellular potential (ψ^{mc}) and transepithelial current (I_T). After baseline measurements were obtained, serosal Cl^- had been replaced with gluconate 51 min before the time of applying the first pulse train of the Figure. The anionic replacement produced a progressive fall in short-circuit current (I_{sc}) and membrane depolarization. Upon restoring Cl^- to the serosal solution, the intracellular potential became more negative and I_{sc} increased. The data of Figs. 3–5 were obtained with apical impalements of the same full-thickness preparation

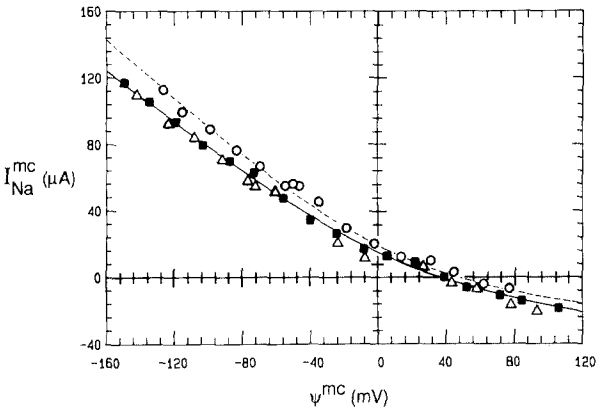

Fig. 4. Fits of the constant field equation to the relationship of Na^+ current to membrane potential across the apical membrane before, during and after replacing serosal Cl^- with gluconate. The data points symbolized by squares and triangles were obtained before and after Cl^- replacement, respectively. These two sets of data points are congruent, and are fit by the Goldman equation (solid line) with $P^{ap}_{Na} = 17.2 \times 10^{-7}$ cm^3 · sec^{-1} and $c^c_{Na} =$ 27.6 mM. The set of data points symbolized by circles were obtained and serosal gluconate, and are fit by the Goldman equation when $P^{ap}_{Na} = 19.8 \times 10^{-7}$ cm^3 · sec^{-1} and $c^c_{Na} = 18.4$ mM. The apical membrane potential and short-circuit current were actually sampled 25 times during each pulse train. To avoid obscuring the records, only three points measured under holding conditions are included for each graph: the two extreme values and the mean of the 25 measurements

full-thickness skins of the current work. In only two of 10 such anionic replacements was I_{sc} clearly reduced. On the other hand, in each case, restoration of serosal Cl^- uniformly produced transient increases in the short-circuit current and transepithelial conductance (Fig. 3). Despite these relatively modest effects on I_{sc} and g_T, the serosal Cl^- substitution exerted marked effects on the membrane potential of each of the preparations studied. In six technically satisfactory experiments, gluconate replacement of serosal Cl^- depolarized the apical membrane, whether or not the short-circuit current was depressed. The mean depolarization was 21 ± 3 mV (Table 2). Figure 3 illustrates the changes triggered by restoring serosal Cl^-: hyperpolarization of ψ^{mc} (given by the lower envelope of the upper trace), increase in the fractional resistance across the apical membrane (proportional to the deflections of the upper trace), increase in I_{sc} (the upper envelope of the lower curve), and increase in the transepithelial conductance (proportional to the deflections of the lower trace). The interposed dark triangles reflect the application of the trains of voltage pulses.

Figure 4 presents data (from the same skin) reduced from the current-voltage relationships measured during the application of three trains of voltage pulses. The squares and triangles symbolize data points obtained before and after the Cl^- replacement, respectively. These two sets of data are well fit by the same theoretical plot (solid line), calculated from Eq. (1). The data points symbolized by the circles were obtained during the period of Cl^- replacement, and are well fit only when distinctly different values for P^{ap}_{Na} and c^c_{Na} are inserted in Eq. (1) (interrupted curve). From these fits to the Goldman equation, we calculate that serosal Cl^- replacement reduced c^c_{Na} from 28 to 18 mM, while increasing P^{ap}_{Na} from 17 to 20 $\times$ 10^{-7} cm^3 · sec^{-1}.

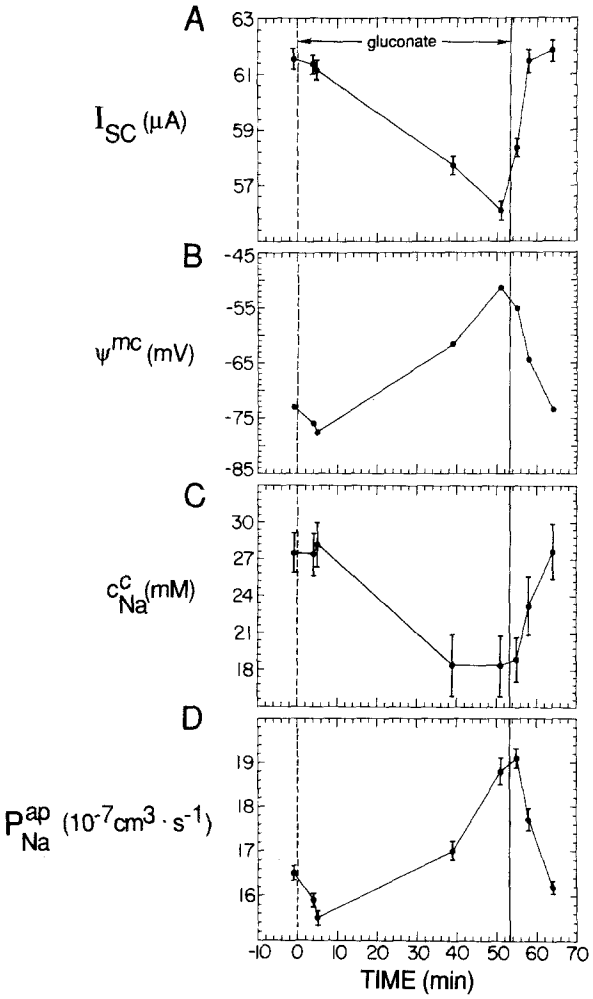

Fig. 5. Time course of effects of serosal Cl^- replacement on I_{sc}, ψ^{mc}, c^c_{Na} and P^{ap}_{Na}. The error bars represent ±1 SE. The mean values of ψ^{mc} in the short-circuited state were sampled 25 times during each train of voltage pulses, so that the SE is included within the symbol for each data point. Gluconate replaced Cl^- in the serosal perfusion solution at time 0 (interrupted vertical line), triggering a marked depolarization of the apical membrane, reductions of intracellular Na^+ concentration and short-circuit current, and an increase in apical Na^+ permeability. All of the changes were reversed by restoring Cl^- to the serosal medium 53 min later (solid vertical line)

The time courses of the changes in I_{sc}, ψ^{mc}, c^c_{Na} and P^{ap}_{Na} are presented in Fig. 5 for the same preparation as that of Figs. 3 and 4. This Figure more clearly documents an increase in apical Na^+ permeability and a fall in intracellular Na^+ concentration in the preparation studied. This observation has at least three implications. First, Cl^- replacement could not have inhibited Na^+ transport by a primary action to block the apical Na^+ channels. Second, the data are incompatible with a substantial primary effect of Cl^- replacement to inhibit the basolateral Na,K-exchange pump; otherwise, the pump inhibition and enhanced P^{ap}_{Na} would have led to an increased intracellular Na^+ concentration, contrary to observation. Finally, the Cl^--dependent membrane depolarization could not have reflected increased Na^+ entry through basolateral conductive channels; in that event, too, c^c_{Na} should have risen.

The averaged results for the series of apical impalements are presented in Table 2. Since gluconate replacement of serosal Cl^- increased P^{ap}_{Na} in each experiment, the probability of the null hypothesis is $(0.5)^6 < 0.02$. The fall in intracellular Na^+ concentration was not statistically significant for the combined series of experiments.

Discussion

The electrophysiologic measurement of apical Na^+ permeability and intracellular Na^+ concentration permits the rapid monitoring of these parameters in frog skin. In the present study, each experimental determination could be completed within 6.1 sec, providing the opportunity to follow the time courses of changes in P^{ap}_{Na} and c^c_{Na}, as well as in ψ^{mc} and I_{sc} (Fig. 5). In principle, analysis of the brief pulses used could be obscured by capacitative transients (Garcia-Diaz & Essig, 1985). However, as previously reported (DeLong & Civan, 1984), varying the sampling time within a 31-msec period after initiating the pulses had little effect on the estimated values of P^{ap}_{Na} and c^c_{Na}. In the present work, we have extended that range of sampling times. The calculated values of both parameters have been little changed when the pulse duration has been varied over the range 16 to 80 msec (Table 1). Furthermore, the asymmetrical transient responses of transepithelial current-to-voltage pulses sometimes observed (Fig. 1) has suggested that some of the time-dependent changes may reflect polarization effects noted with longer pulses (Weinstein et al., 1980), and not solely reflect capacitative transients. In studying the effects of Cl^- on Na^+ transport, we have therefore chosen to use voltage pulses of 16- to 32-msec duration. A similar approach has been taken by Schoen and Erlij (1985), who have conducted measurements of the apical membrane at intervals of 20 and 60 msec after applying the transepithelial voltage pulse.

Substitution of serosal Cl^- by gluconate has been reported to reduce short-circuit current across both frog skin (Duffey et al., 1986) and toad urinary bladder (Lewis et al., 1985). In principle, the inhibition could reflect one or more primary actions to: (i) decrease the apical Na^+ permeability, (ii) decrease the electrical and/or concentration driving forces for apical Na^+ entry from the mucosal medium into

the cell, and (iii) decrease the rate of Na^+ extrusion from the cell into the serosal medium by the Na,K-exchange pump. In view of the importance of apical Na^+ permeability in the physiologic regulation of Na^+ transport across tight epithelia (Lichtenstein & Leaf, 1965; Lewis et al., 1976; Civan, 1983), we have measured P_{Na}^{ap} during the temporary replacement of serosal Cl^-. In the course of these measurements, I_{sc}, c_{Na}^{c} and ψ^{mc} were also monitored in both whole- and split-skin preparations.

In each of the skins studied, gluconate substitution for Cl^- depolarized the apical membrane, reducing the electrical force driving Na^+ entry. In the case of the isolated epithelia, the anionic substitution led to no significant change in P_{Na}^{ap} (Table 2). With continued basolateral extrusion of Na^+, the intracellular Na^+ concentration fell, leading to a lower steady-state rate of Na^+ pump activity across the split skins. In the case of the whole-skin preparations, the gluconate-induced membrane depolarization was associated with a significant increase in P_{Na}^{ap}. Thus, despite the reduced electrical driving force, c_{Na}^{c} was not significantly reduced in that series of tissues, and I_{sc} was consequently little changed. We conclude from these data that gluconate replacement of serosal Cl^- causes a highly reproducible reduction in the electrical force driving apical Na^+ entry, and does not decrease apical Na^+ permeability, whether or not I_{sc} is inhibited.

The basis for the difference in the responses of the split- and full-thickness skins of the present study is unclear. The different responses are unlikely to reflect a fundamental difference in the transport properties of the two preparations, since: (i) the electrophysiological properties of the whole skin and isolated epithelium are similar (Fisher et al., 1980); (ii) I_{sc} across the whole skin was at least occasionally inhibited by removing serosal Cl^- (Fig. 5); and (iii) in each experiment, the restoration of Cl^- produced a transient stimulation of short-circuit current (as well as a repolarization of the apical membrane) (Fig. 4).

An alternative basis for the different responses of I_{sc} may be the slow exchange in the whole-skin preparation, between the bulk medium and the extracellular fluid in contact with the basolateral membranes. Thus, removal of Cl^- from the bulk serosal medium may have been less effective in reducing the Cl^- concentration (c_{Cl}^{bl}) at the basolateral surface of the full-thickness preparation. Consistent with this view is the observation that the membrane depolarization was only about half as great in the whole skins (21 ± 3 mV) as in the split skins (37 ± 4 mV) (Table 2). Gluconate substitution for Cl^- may result in more than one effect on Na^+ transport. In addition to reducing the electrical driving force for Na^+ entry, a compensatory increase in P_{Na}^{ap} can evidently be induced under certain conditions (Table 2). In addition, Lewis et al. (1985) have noted suggestive evidence that the anionic substitution can lead to a reduction in the basolateral pump density of toad bladder (their p. 134). The concentration dependences of these multiple effects have not been defined, and may substantially differ from one another.

This putative slow rate of exchange between the bulk medium and the interstitial fluid adjacent to the basolateral membranes may have also played a role in other published studies. For example, Biber et al. (1985) reported that unilateral substitution of methanesulfonate for Cl^- produced no significant immediate change in either ψ^{mc} or I_{sc} across whole frog skin. In addition, Lewis et al. (1985) have reported that as long as ~50 min was sometimes necessary before a reduced steady-state level of I_{sc} was reached after gluconate substitution for serosal Cl^- (their Fig. 5). The latter authors suggested that the prolonged lag time reflected a period required to trigger an intermediate series of intracellular events.

Whether access from the bulk medium to the epithelial surface was rapid (as in the split skins) or slow (as in the whole skins), the single most striking effect of the serosal Cl^- replacement was the intracellular depolarization. In principle, this effect could have reflected: a Cl^- diffusion potential, inhibition of the electrogenic Na^+ pump, an increase in either apical or basolateral Na^+ conductance, or a reduction in the basolateral K^+ permeability. Two considerations indicate that the depolarization did not reflect an altered diffusion potential through basolateral Cl^- channels: (i) The basolateral Cl^- conductance of the major cell population of frog skin is likely very low (Biber et al., 1985); and (ii) The time course is inappropriate. The depolarization is slowly progressive and sustained, persisting even 4 hr after serosal Cl^- removal (Duffey et al., 1986), in contrast to the transient depolarization associated with the leaching of Cl^- out of other cells (Vaughan-Jones, 1979; Aicken & Brading, 1984).

The Cl^--dependent depolarization also did not reflect inhibition of the Na,K-exchange pump. Such inhibition would have led to an increase in c_{Na}^{c}, contrary to the decreased concentration noted in each of the split-thickness preparations and in half of the full-thickness skins. Finally, the depolarization cannot have resulted solely from the increased apical Na^+ permeability observed with the whole thickness preparations. The depolarizations observed during the basolateral penetrations of the split-thickness skins were even larger, but were unassociated with a significant increase in P_{Na}^{ap} (Table 2).

By exclusion, it appears likely that the depolarization results from an inhibition of the basolateral K^+ conductance triggered by serosal Cl^- removal. The depolarization can reduce apical Na^+ entry, and can lead to a reduction in intracellular Na^+ concentration, thereby presenting the pump with a lowered rate of Na^+ substrate delivery.

This conclusion is in agreement with that of Lewis et al. (1985), who have presented more direct evidence that gluconate replacement for serosal Cl^- reduces P_K^{bl} in toad bladder. They observed that the serosal gluconate substitution prevented Ba^{2+} from further inhibiting I_{sc} and reducing the estimated cell electromotive force. In addition, transepithelial measurements of bladders exposed to mucosal nystatin suggested that the relative basolateral permeability of Na^+ to K^+ was an order of magnitude larger in the presence of external gluconate than external acetate. (In contrast to the inhibitory effect of serosal gluconate, acetate replacement of serosal Cl^- stimulated I_{sc}.)

The signal transduction by which replacement of serosal Cl^- by gluconate reduces basolateral K^+ conductance is unknown. Cell volume, pH and intracellular Ca^{2+} activity have been considered to possibly play mediating roles (Civan, 1986). External Cl^- replacement does alkalinize the epithelial cells of frog skin (Civan & Peterson-Yantorno, 1986; Duffey et al., 1986). However, the reduction in short-circuit current is not solely a direct result of the sustained intracellular alkalinization. Administration of 1 mM DIDS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid) blocks the sustained intracellular alkalinization characteristically produced by Cl^- replacement, but does not affect the inhibition of Na^+ transport (Civan & Peterson-Yantorno, 1986. Both the changes in transepithelial ion transport and in intracellular pH may be triggered by the reduction in cell volume resulting from the placement of serosal Cl^- by a poorly permeable anion. When the magnitude of the Cl^--dependent change in cell volume is minimized in toad urinary bladder (Lewis et al., 1985) and in frog skin (Civan & Peterson-Yantorno, 1987), the fall in short-circuit current is reduced, and the alkalinization is also decreased (Civan & Peterson-Yantorno, 1987). The precise coupling between volume and basolateral K^+ conductance is unclear. However, such coupling is consistent with observations in other cells (Davis & Finn, 1985; Hoffman, 1985; Germann, Ernest & Dawson, 1986), and provides a mechanism for regulating cell volume (Schultz, 1981). During cell shrinkage, closure of K^+ channels will reduce the rate of loss of K^+ solute down its electrochemical gradient, thereby conserving intracellular solute.

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