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Influence of Serosal Cl on Transport Properties and Cation Activities in Frog Skin

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Summary. The effects of serosal substitution of isosmotic Na_2SO_4-Ringer solution for NaCl-Ringer solution were studied in the short-circuited frog skin (*Rana pipiens*, Northern variety). Despite prompt changes of transepithelial measurements, initial cellular effects were slight. After 30 to 45 min, however, the transcellular current had decreased and the cell electrical potential had depolarized, in association with decrease of the apical membrane fractional resistance and basolateral membrane conductance. Apical membrane slope conductance was unaffected. Similar effects were obtained with isolated epithelia. With the use of gluconate or NO_3 in place of Cl, the effects on cellular current and conductance were minimal or insignificant, despite changes of the cell potential, fractional resistance, and basolateral conductance similar to those seen with sulfate. Following prolonged exposure to serosal SO_4-Ringer, the extent of depolarization induced by raising the serosal K concentration decreased, indicating diminution of basolateral K conductance and the existence of other basolateral conductances. Equilibration in serosal gluconate-Ringer enhanced polarization on serosal restoration of Cl or removal of Na, again indicating a time-dependent change in the basolateral conductance pattern. Depolarization on removal of serosal Cl was not attributable to inhibition of the pump. Nor was it the result of decrease of the K equilibrium potential E_K: exposure to serosal SO_4-Ringer decreased cell K activity a_K^c from 104 ± 6 to 58 ± 4 mM (n = 5), but E_K was reduced only slightly; exposure to serosal gluconate increased a_K^c and E_K. Serosal sulfate lowered the cell Na activity a_{Na}^c, but the electrochemical potential difference for Na across the apical surface was unaffected. The concurrent decrease of both a_K^c and a_{Na}^c following serosal substitution of SO_4 for Cl raises questions concerning mechanisms of osmoregulation.

Key Words frog skin · microelectrodes · K activity · Na activity · membrane conductance · chloride

Introduction

We have reported that bilateral replacement of chloride by sulfate caused marked inhibition of transepithelial Na transport and depolarization of the cell potential in the short-circuited frog skin (Klemperer et al., 1986*b*). Similar effects have been observed in split frog skins when serosal Cl was replaced by either SO_4 or gluconate (DeLong & Civan, 1984; Giraldez & Ferreira, 1984). Changes in cell ion and water content following serosal Cl replacement by gluconate have been studied with isotopic and chemical techniques by Ferreira and Ferreira (1981), with ion-selective electrodes by Giraldez and Ferreira (1984) and by Duffey et al. (1986), and with electron microprobe analysis by Dörge et al. (1985).

To extend and clarify previous observations, microelectrodes were used to monitor the intracellular electrical potential and K and Na activities, and their responses to serosal Cl substitution. We found that the depolarization induced by replacement of serosal Cl by SO_4, gluconate, or NO_3 was associated with diminution of basolateral conductance. These effects were seen irrespective of whether cell K activity decreased (in SO_4-Ringer) or increased (in gluconate-Ringer). The altered basolateral conductance pattern was not mediated by effects on the pump.

Part of this work has been presented in an abstract (Klemperer, García-Díaz & Essig, 1986*a*).

Materials and Methods

Sections of abdominal skins of *Rana pipiens pipiens*, Northern variety, (Connecticut Valley Biological or Kons, Germantown, WI) were mounted horizontally (mucosa up) in an Ussing-type chamber (0.36 cm^2), as previously described (Klemperer et al., 1986*b*). Isolated epithelia were obtained according to the method of Cox and Helman (1983).

The serosal surface of the tissues was perfused with NaCl-Ringer solution (NaCl-R) containing (in mM): 110 NaCl, 1 $CaCl_2$, and 2.5 KOH, and titrated to pH 7.8 with HEPES. In ionic substitution experiments, NaCl was replaced by (in mM) 55 Na_2SO_4, 55 K_2SO_4, 110 $NaNO_3$, 110 Na gluconate, 110 N-methyl-D-glucamine (NMDG) Cl, 110 NMDG gluconate, or 55 $(NMDG)_2SO_4$. The osmolality of the solutions (measured by a Precision System osmometer) was 220 mOsm/Kg H_2O, adjusted

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when required by addition of mannitol. Unless otherwise indicated, $NaNO_3$ replaced NaCl in the mucosal Ringer solution, in order to minimize paracellular conductance (García-Díaz et al., 1985). Amiloride (Merck, Sharp and Dohme, West Point, PA) was added to the mucosal bath to a final concentration of 2×10^{-5} M and ouabain (Sigma) was added to the serosal bath to a final concentration of 10^{-4} M.

Microelectrodes were drawn from self-filling borosilicate tubing (Kwikfil 1B 120F, WP Instruments), using a horizontal puller (Industrial Science Assoc., Model 1). Open-tip microelectrodes were filled with 1.5 M KCl, giving resistances exceeding 30 MΩ. For preparing ion-selective electrodes, the pulled capillaries were exposed to bis-(dimethylamine)-dimethylsilane (Fluka) vapors under vacuum for 4 min and then heated on a hot plate (400°C) for an hour. Potassium ion exchanger (Corning 477317) or sodium ion exchanger (ETH 227, Sodium-Ionophore I, Fluka) was introduced into the back of the pipette, filling the tips in most cases within one-half hr. Filling was then completed with 0.5 M KCl or 0.2 M NaCl, respectively.

The transepithelial electrical potential V_t and the apical membrane potential V_o were measured as described previously (Klemperer et al., 1986*b*). The use of flowing KCl bridges between the calomel electrodes and the bathing solutions minimized junction potentials. Tissues were short circuited except for intermittent voltage clamping to +10 mV for 300 msec to measure the transepithelial conductance g_t and the apical membrane voltage-divider ratio ($F_o \equiv \Delta V_o/\Delta V_t$). Occasional exposure to amiloride for one min permitted estimates of the amiloride-sensitive current I_c and conductance g_c.[1] Intracellular ionic activities were measured by the simultaneous use of open-tip and ion-selective microelectrodes, the voltages of each having initially been set to zero in the mucosal solution (García-Díaz et al., 1985). The intracellular voltage difference between the two electrodes then allowed calculation of the cellular K activity a^c_K or Na activity a^c_{Na} by interpolation from the appropriate calibration curve (García-Díaz et al., 1986). Because of nonlinearity of the response at low activities, the use of this technique was particularly important with the Na electrode. The responsivity of the K-selective microelectrodes was 57.9 ± 0.4 mV/decade and the K/Na selectivity ratio was 38.7 ± 3.3 (n = 16). Activity coefficients of Na and K were 0.77 in 110 mM chloride solutions and varied little with the substitution of NO_3 or gluconate for Cl; in the corresponding SO_4 solutions values for Na and K were 0.47 and 0.43, respectively.

Impalements were across the mucosal surface, with the electrodes mounted on stepping motor manipulators (E. Nagel, Biomedizinische Instruments, Munich, FRG, Model MF-500) almost perpendicular to the skin. Our technique of impalement and criteria for validating intracellular measurements are described elsewhere (García-Díaz et al., 1985; Klemperer et al., 1986*b*; García-Díaz et al., 1986). Briefly, following impalement, open-tip electrodes were withdrawn in 0.5 μm steps in order to reduce R^{el} towards the level measured prior to cell puncture. Impalements were accepted only if readings were stable following application of mucosal amiloride or removal of mucosal sodium, with V_o more negative than −100 mV and $F_o > 0.95$ (in serosal NaCl-R). Changes in tip potential of up to 3 mV were permitted. Impalements with ion-selective electrodes were accepted only if values of F_o measured simultaneously with open-tip and ion-selective electrodes agreed within 3%, and if the electrode difference signal was constant (±3 mV) 30–60 sec after application of amiloride. Solutions were changed without loss of impalement by use of a magnetic valve (mucosal) or a noninterrupting valve (serosal). Unless stated otherwise, results are given as the mean ±SEM. Significance was tested using a paired Student's t test with a level of significance of 0.05.

[1] Mixing in our chamber was not sufficiently good to assure complete effects on I and g within the 1 min of exposure to amiloride. Longer exposure was avoided in order to speed recovery following removal of amiloride, so as to minimize the likelihood of dislodgement of the microelectrode and permit observations on the same cell before and after the change of media. Change of paracellular conductance following the application of amiloride contributes inaccuracy to the evaluation of I_c and g_c. This effect is minimized by the substitution of NO_3 for mucosal Cl and the avoidance of sustained perturbations of V_t (Nagel, García-Díaz & Essig, 1983, 1988).

Table of Main Symbols Used

$a_{K(Na)}$	potassium (sodium) activity (mM)
E_K	potassium equilibrium potential (mV) across the inner (basolateral) membrane
$E_{Na,o}$	sodium equilibrium potential (mV) across the outer (apical) membrane
F_o	voltage-divider ratio across the outer (apical) membrane
g	slope conductance (mS/cm²)
g_i	inner (basolateral) membrane slope conductance (mS/cm²), calculated according to Frömter and Gebler (1977): $g_i = (g - g_p)/(F^A_o - F_o)$
G	chord conductance (mS/cm²)
G_i	inner (basolateral) membrane chord conductance (mS/cm²), calculated at short circuit from changes induced by amiloride: $G_i = \Delta I_c/\Delta V_o$
g_o	outer (apical) membrane slope conductance (mS/cm²): $g_o = 1/(1/g_c - 1/g_i)$
I	current (μA/cm²) from outer to inner solution
R_{el}	microelectrode resistance (MΩ)
$V_{K(Na)}$	voltage between the K(Na) sensitive electrode and mucosal bath (mV)
V	potential (mV)

V_o and V_t are expressed with reference to the mucosal bathing solution.

Subscripts: t, c, and p refer to transepithelial, cellular (amiloride-sensitive), and paracellular (amiloride-insensitive) pathways, respectively; o and i refer to the outer (apical) and inner (basolateral) cell boundaries. The superscript A denotes a measurement in the presence of 2×10^{-5} M mucosal amiloride.

Results

Substitution of Serosal Cl-R by SO_4-R

We have reported that the apical membrane potential of the short-circuited frog skin is depolarized by the bilateral substitution of Na_2SO_4-R for NaCl-R (Klemperer et al., 1986*b*). Similar effects were now

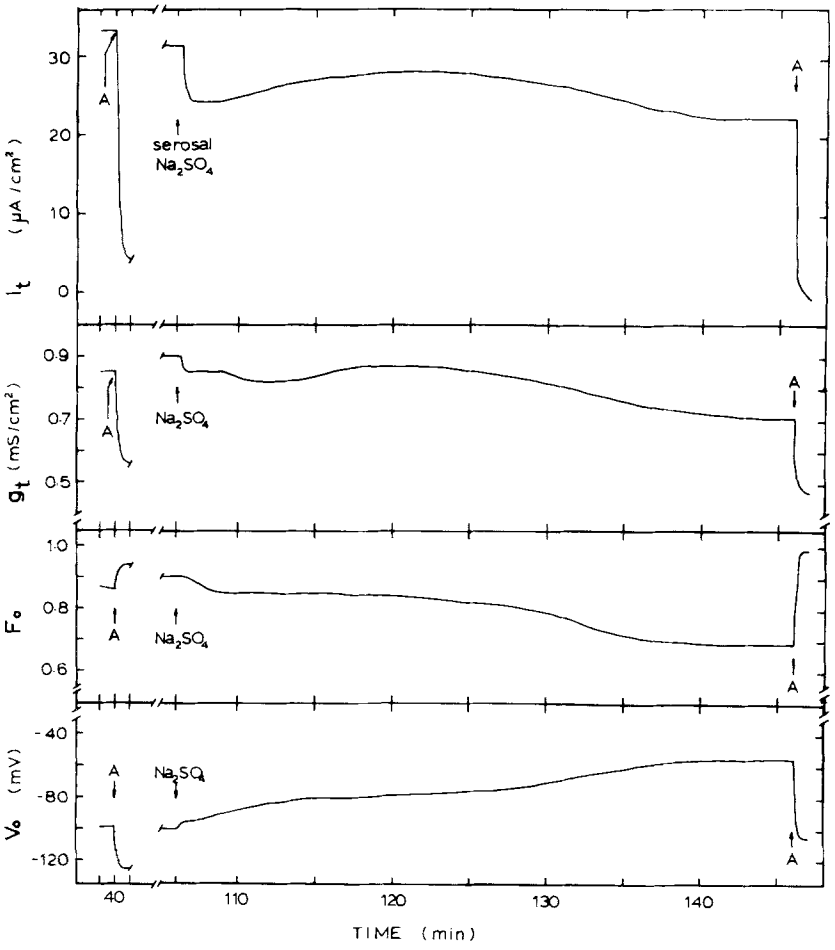

Fig. 1. Effects of replacement of serosal NaCl-R with Na_2SO_4-R. The short-circuited skin was equilibrated in NaCl-R solutions bilaterally. Amiloride was administered in order to verify the validity of the impalement and evaluate cellular and paracellular parameters. After recovery from amiloride, serosal NaCl-R was replaced by Na_2So_4-R. After 40 min, amiloride was again administered

found following serosal replacement of NaCl-R by Na_2SO_4-R (Fig. 1). Although there was prompt decrease of the transepithelial current I_t and conductance g_t, initial effects on the cell potential V_o and voltage-divider ratio F_o were slight. During the subsequent 30 min there were biphasic changes in I_t and g_t, followed by progressive decrease of I_t and g_t, depolarization of V_o, and decline of F_o. Brief exposure to amiloride demonstrated that following 30 min of exposure to serosal SO_4-R the amiloride-sensitive current I_c (presumably representing transcellular Na transport) had decreased.

Because the initial decrease of I_t might possibly have resulted from a transient diffusion potential across the serosal connective tissue, the experiment of Fig. 1 was repeated in five studies using isolated epithelia. As is shown in Fig. 2, the response of I_t was similar to that in the intact skin. Another possible explanation for the prompt decrease of I_t is net anionic current from mucosa to serosa. This is consistent with the results of the experiment of Fig. 3, showing that, unlike the case in the steady state (Fig. 1), the amiloride-sensitive current I_c was unchanged 1 min after substitution of SO_4 for Cl. The

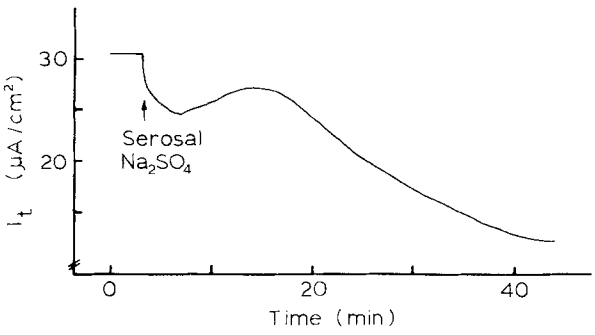

Fig. 2. Effect on short-circuit current of replacement of serosal NaCl-R with Na_2SO_4-R (isolated epithelium)

decrement in I_t observed at this time was entirely attributable to negative current in the amiloride-insensitive (paracellular) pathway, which must have been carried by nitrate.

Figure 4 shows steady-state control and experimental values of electrophysiological variables in seven experiments similar to that of Fig. 1. After 40–50 min in Na_2SO_4-R the inhibition of I_c accounts for the decrease of I_t (Fig. 4*A*). Concomitant de-

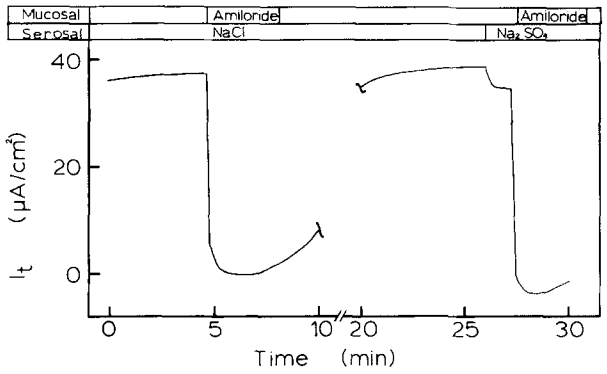

Fig. 3. Early effect on I_c of replacement of serosal NaCl-R with Na_2SO_4-R. After equilibration of the short-circuited skin in NaCl-R, amiloride was applied to allow measurement of I_c. Following removal of the drug and recovery, the serosal solution was replaced by Na_2SO_4-R for 1 min and amiloride was again administered. (One of four similar experiments)

crease of g_t is attributable to lower g_c (Fig. 4*B*). V_o depolarized following substitution of serosal Cl with SO_4, by 43 ± 8 mV with mucosal NaCl-R and by 26 ± 16 mV with mucosal $NaNO_3$-R (Fig. 4*C*). F_o dropped in 6 out of 7 experiments (Fig. 4*D*), reflecting the decrease of the inner membrane conductance g_i to a mean of ~30% of its original level (Fig. 4*E*). In contrast to the pronounced effect on g_i, there was no significant change of the apical slope conductance.

Because of depression of the activity coefficient of K, the substitution of SO_4 for Cl lowered serosal a_K from 1.95 to 1.3 mM. In order to examine the influence of serosal K activity, three experiments were performed in which the Na_2SO_4-R contained 4.3 mM instead of 2.5 mM K, giving a serosal a_K of ~2.6 mM. The effects of 40–50 min of exposure to serosal Na_2SO_4 were similar to those observed pre-

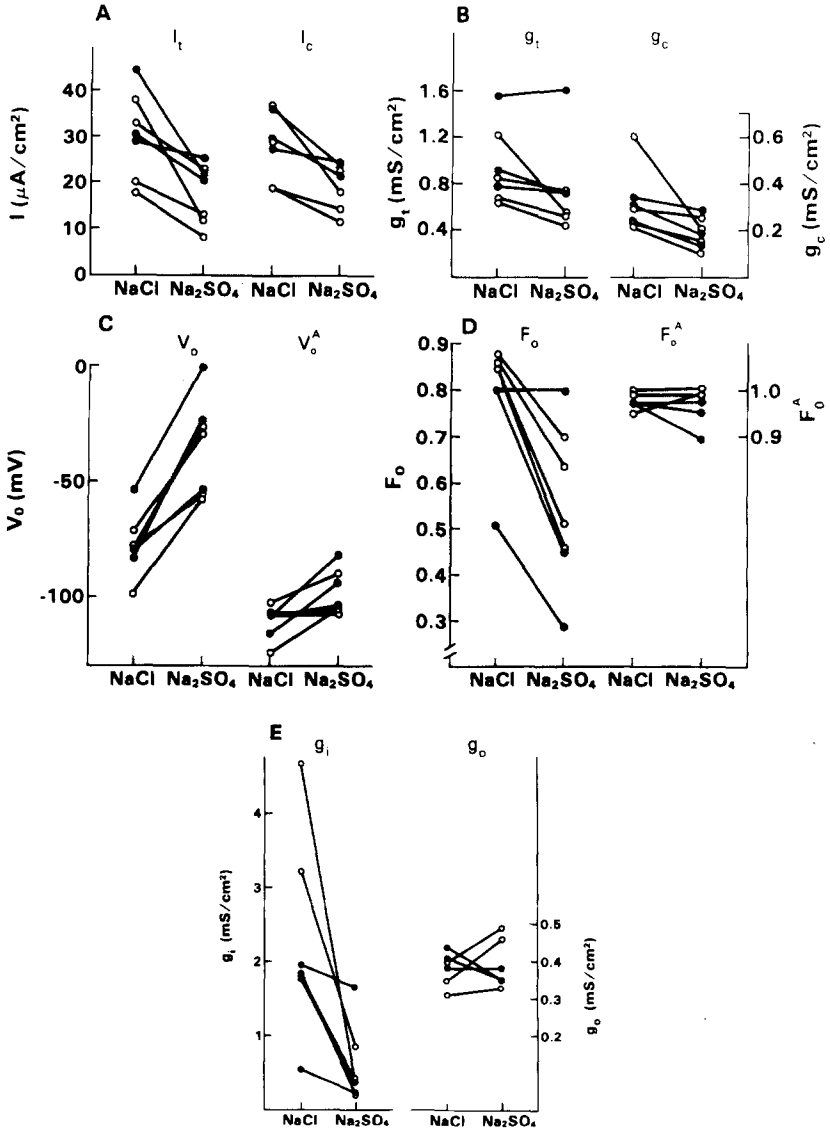

Fig. 4. Steady-state values of electrophysiological variables during serosal exposure to NaCl-R and after 40–50 min of serosal exposure to Na_2SO_4-R (n = 7). Open and filled symbols denote mucosal NaCl-R and $NaNO_3$-R, respectively. Points from the same experiment are joined by a line. Overlapping lines were omitted for clarity. *A*: I_t and I_c (*see* Footnote 1); *B*; g_t and g_c; *C*: V_o and V_o^A; *D*: F_o and F_o^A; *E*: g_i and g_o

viously (Fig. 1), except that depolarization by SO_4-R in the presence of amiloride was more pronounced.

Substitution of Serosal Cl by Gluconate or NO_3

Because the above results do not distinguish between effects of removal of Cl and those of exposure to SO_4, we next studied the effects of replacement of Cl by either gluconate or NO_3 (Fig. 5). Effects on I_t, I_c, g_t, and g_c were insignificant or minimal. As previously, however, V_o and V_o^A were depolarized, and F_o and g_i were lowered, whereas g_o was unaffected.

Substitution of Serosal Cl-R by SO_4-R in the Presence of Amiloride and Ouabain

In order to test whether depolarization was attributable to inhibition of the pump, the response of V_o to the removal of serosal Cl was examined in the absence of net sodium transport. The results of a representative study are shown in Fig. 6. After equilibration in serosal NaCl-R, the short-circuited split skin was exposed to mucosal amiloride. I_t was completely inhibited within 2–3 min and V_o showed typical prompt hyperpolarization followed by progressive depolarization (García-Díaz et al., 1985). The serosal application of ouabain after 10 min of exposure to amiloride did not affect I_t, but caused additional depolarization of V_o. On achievement of a steady state, the serosal NaCl-R was replaced by Na_2SO_4-R. I_t now became negative, most likely because of the large transepithelial NO_3 gradient. V_o promptly depolarized slightly, repolarized, and then depolarized further towards a steady-state value of ~−50 mV. The extent and time course of these effects were similar to those observed previously in the absence of ouabain, indicating that pump inhibition is not the cause of depolarization.

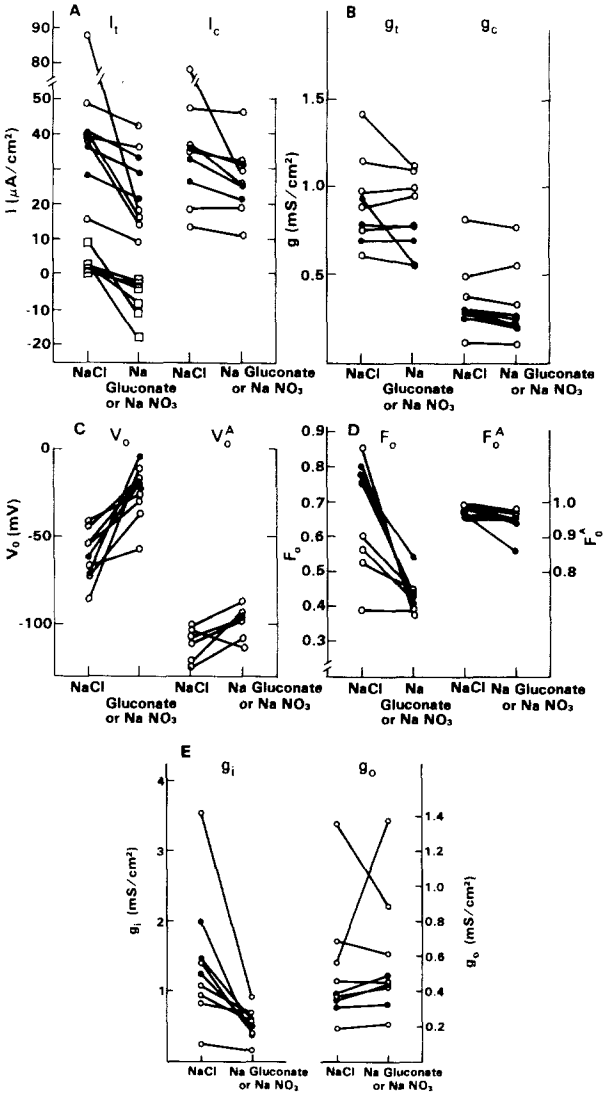

Fig. 5. Steady-state values of electrophysiological variables during serosal exposure to NaCl-R and after 45 min of serosal exposure to Na gluconate-R (open symbols, $n = 6$) or $NaNO_3$-R (filled symbols, $n = 3$). Points from the same experiment are joined by a line. Overlapping lines were omitted. *A*: I_t (○–○), I_c (○–○), and I_p (□–□); *B*: g_t and g_c; *C*: V_o and V_o^A; *D*: F_o and F_o^A; *E*: g_i and g_o

Depolarization by Serosal K

In order to characterize the basolateral K conductance in Cl-free media, the serosal K concentration $[K]_s$ was elevated in the absence of significant transcellular transport. If selectivity for K were complete, K should rapidly come to thermodynamic equilibrium across the basolateral membrane. This would require that V_o rapidly increase to the same extent as the potassium equilibrium potential E_K, namely, for the increase of $[K]_s$ from 2.5 to 112.5 mEq/liter employed here, by ~98–99 mV. This was not found. In the study of Fig. 7*A*, after the short-circuited skin was equilibrated with serosal NaCl-R, followed briefly by serosal Na_2SO_4-R, the cellular current was inhibited by amiloride. Replacement of the serosal solution by K_2SO_4-R then caused a depolarization of 85 mV. After reequilibration with NaCl-R the procedure was repeated, but with longer exposure to Na_2SO_4-R (Fig. 7*B*). The depolarization was now only 71 mV. In three such experiments, the mean depolarization induced by high $[K]_s$ following Cl replacement was 85 ± 1 after 7–9 min and 66 ± 3 mV after 50 min. These findings indicate basolateral conductance in addition to that

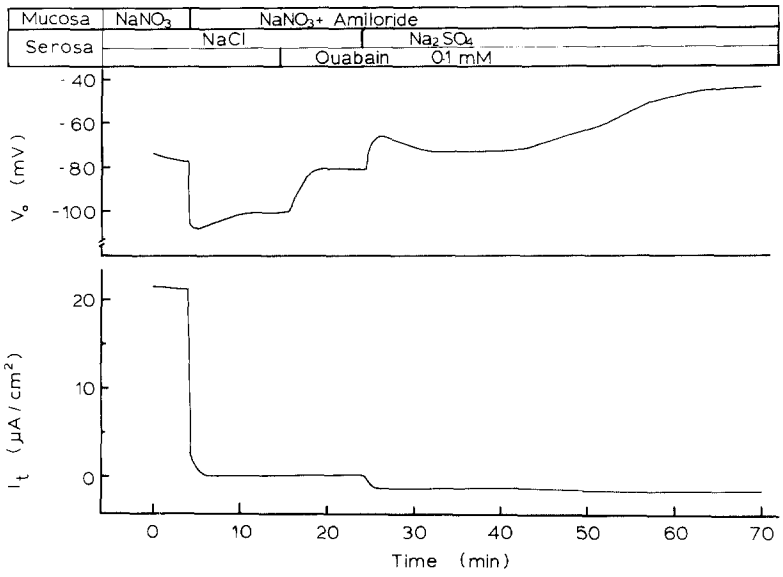

Fig. 6. Lack of influence of pump function on depolarization of V_o by serosal Na_2SO_4-R. Short-circuited split skins were equilibrated with serosal NaCl-R. Amiloride was then added to the mucosal solution. Following the usual response of V_o to amiloride, ouabain was added to the serosal solution in order to block the Na pump. The serosal solution was then replaced by Na_2SO_4-R for 45 min

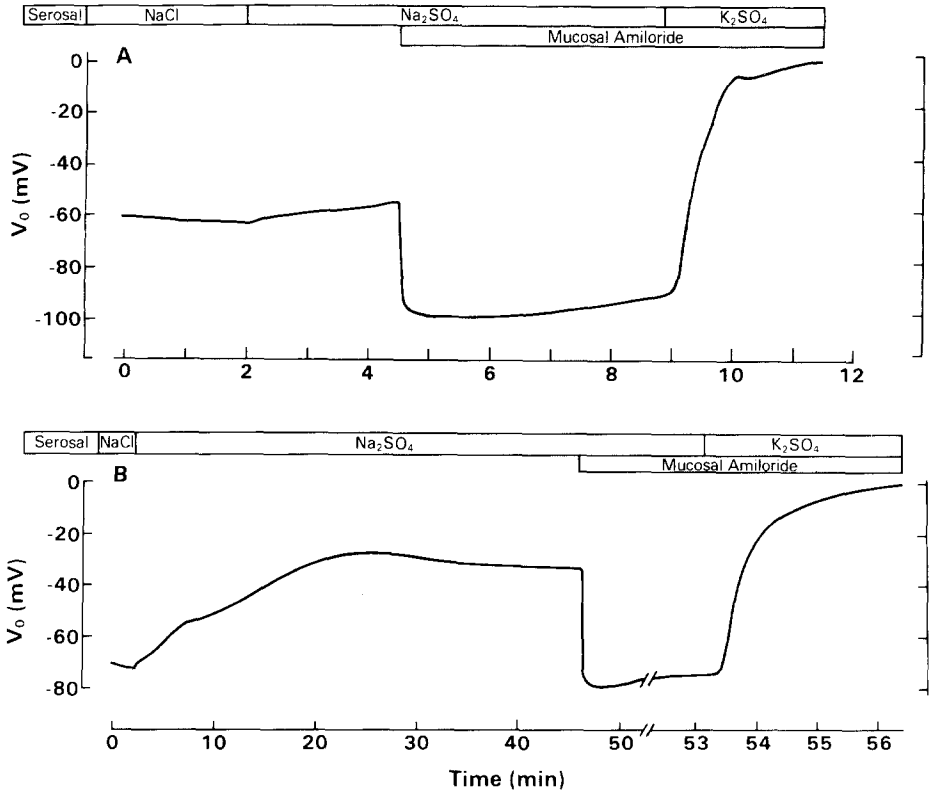

Fig. 7. Effect of prolonged exposure to serosal Na_2SO_4-R on response of V_o to high $[K]_s$. The short-circuited skin was first equilibrated with serosal NaCl-R. (*A*) The serosal solution was replaced by Na_2SO_4-R. About 2 min later amiloride was administered. After an additional 5 min the serosal solution was changed to K_2SO_4-R. V_o depolarized by 87 mV. (*B*) After reestablishing control conditions in the same skin, serosal NaCl-R was replaced with Na_2SO_4-R. Amiloride was added after 45 min. Seven min later the skin was again exposed to serosal K_2SO_4-R. V_o then depolarized by only 71 mV

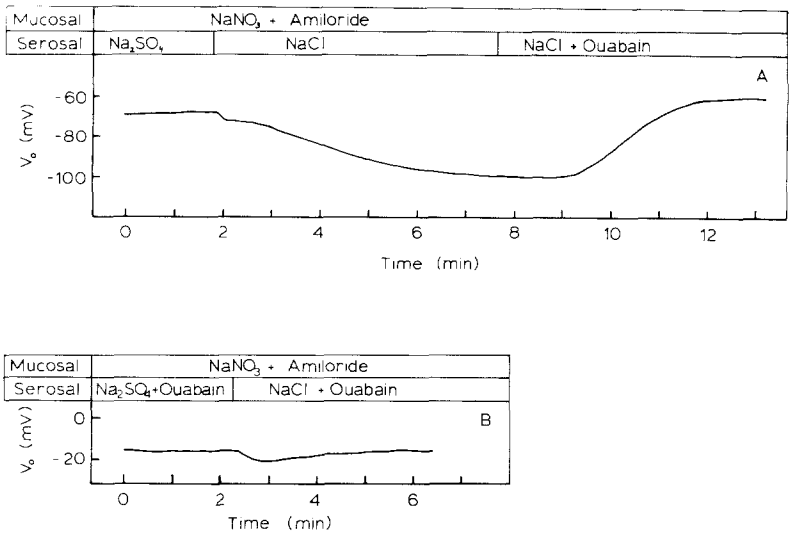

Fig. 8. Influence of pump on response to restoration of serosal Cl. (*A*) The short-circuited skin was first equilibrated with serosal NaCl-R and mucosal $NaNO_3$-R (*not shown*). Amiloride was subsequently administered throughout the remainder of the experiment. After 18 min of exposure to amiloride, serosal NaCl-R was replaced by Na_2SO_4-R for 70 min. (The trace starts towards the end of this period.) At steady state, the serosal solution was changed back to NaCl-R, and after V_o reached a stable value serosal ouabain was administered in order to inhibit the Na pump (*B*). The short-circuited skin was equilibrated as in (*A*). At steady state, ouabain was added to the serosal Na_2SO_4-R. The serosal solution was then replaced by NaCl-R containing ouabain

of K, of increasing importance on prolonged replacement of serosal Cl by SO_4.

Basolateral Conductance Pattern

The slight initial change of V_o with serosal Cl replacement (Fig. 1) suggests that basolateral Cl conductance is small. The issue is confused, however, by the effect of divalent SO_4 on ionic activity coefficients. Furthermore, the major role of Cl in volume regulation suggests possibly important modification of Cl conductance on change of the medium. Ussing (1982) has proposed that Cl channels open when principal (Na transporting) cells lose Cl after osmotic swelling or depolarization. Figure 8 shows that this is not the case with exposure to serosal Na_2SO_4-R. Prior to the beginning of the trace, short-circuited skins in control solutions were treated with amiloride to inhibit Na transport, and exposed to serosal Na_2SO_4-R for 70 min. At steady state, V_o was ~ -65 mV. On then restoring serosal NaCl-R (Fig. 8*A*), V_o hyperpolarized gradually, reaching a value of -100 mV after 5 min. However, the administration of serosal ouabain depolarized V_o to -60 mV. In the experiment of Fig. 8*B*, with preincubation in ouabain, the polarization induced by restoration of NaCl-R was transient and slight; in three tissues the maximal changes were 6, 5, and 6 mV. These observations indicate that the increased negativity of V_o after restoring serosal Cl was largely due to activation of the rheogenic pump. Accordingly, further ionic substitution experiments were carried out in the presence of both amiloride and ouabain. Also, because of the confusing influence of changing activity coefficients, we examined the effects of exchanging Cl and the monovalent ion gluconate.

Figure 9*A* shows the results of a study in which the serosal substitution of gluconate for Cl depolarized V_o by ~ 11 mV. In two similar experiments, however, depolarization was slight or absent. Following equilibration in serosal gluconate, the situation was different (Fig. 9*B*); in 3 studies the substitution of Cl for gluconate increased polarization by 20, 14, and 30 mV, suggesting that under these conditions Cl conductance is significant.

Similar studies examined basolateral Na conductance. Following equilibration in serosal NaCl-R, lowering $[Na]_s$ to 7.5 mM by replacement of Na with NMDG slightly increased membrane polarization (Fig. 10*A*); in four studies the peak change of V_o was 6 ± 1 (SD) mV. Following equilibration in serosal Na gluconate-R, the effects of the same maneuver were more pronounced (Fig. 10*B*); in five studies the peak change was 17 ± 7 (SD) mV. In three tissues equilibrated in SO_4-R, there was also evidence of Na conductance; lowering $[Na]_s$ to 2.2 mM by NMDG replacement increased membrane polarization by 12 ± 8 (SD) mV.

Effects on Cell Activities

Anionic substitution might have depolarized the cell not only by affecting basolateral conductances, but also by decreasing a_K^c, and thus the potassium equilibrium potential E_K. This possibility was tested by the use of K-selective electrodes. Figure 11 shows the effects of serosal Na_2SO_4-R on V_o and the elec-

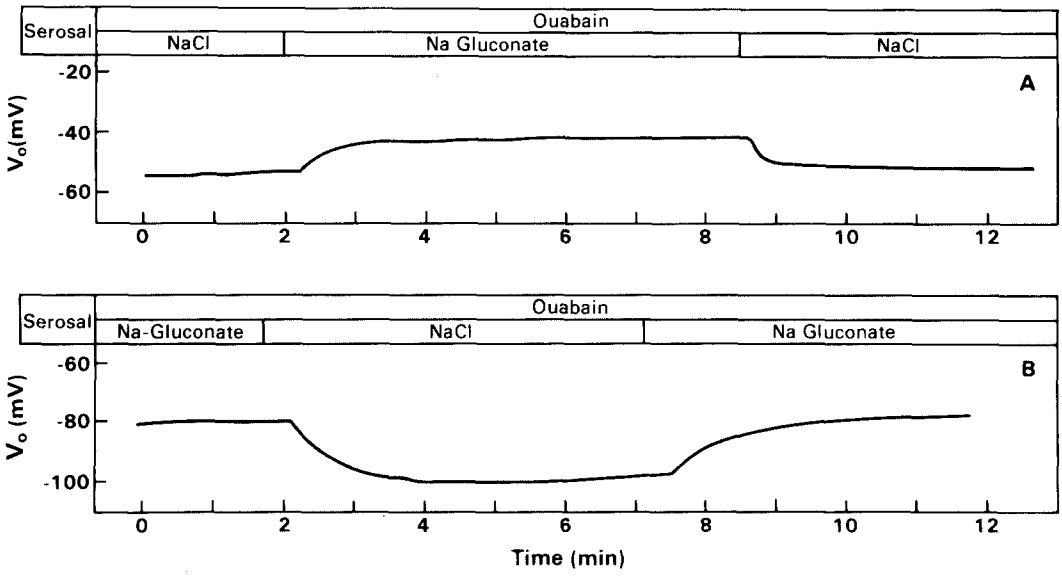

Fig. 9. Influence of serosal incubation medium on response to anionic substitution. Short-circuited skins were incubated with serosal NaCl-R (*A*) or Na gluconate-R (*B*) for over an hour in the presence of amiloride. Ouabain was then administered, followed in an hour by (*A*) brief replacement of serosal NaCl-R with Na gluconate-R or (*B*) brief replacement of serosal Na gluconate-R with NaCl-R

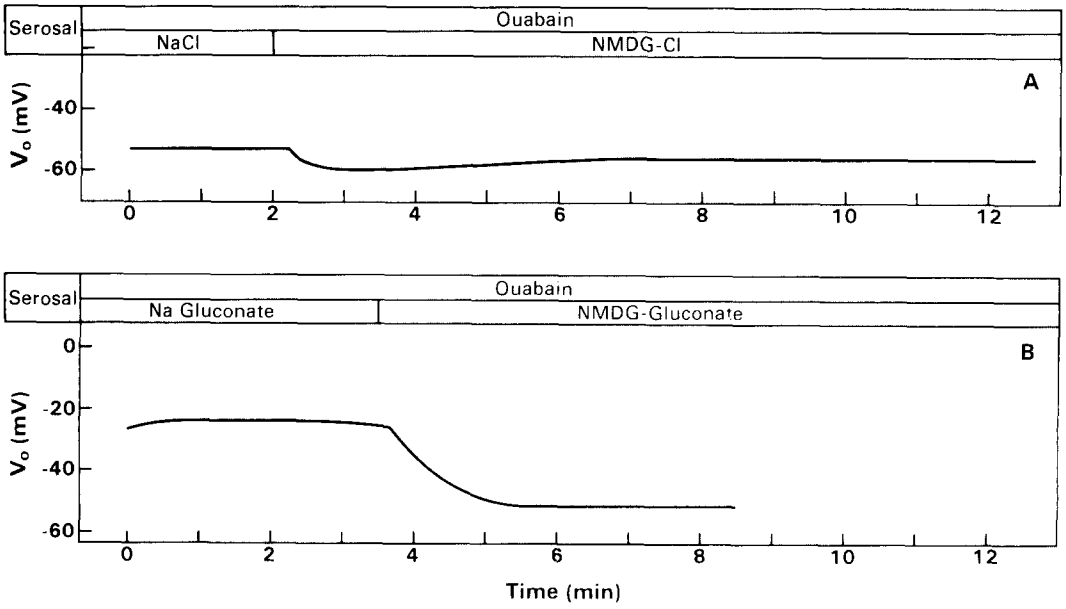

Fig. 10. Influence of serosal medium anion composition on response to Na substitution. Short-circuited skins were equilibrated with serosal NaCl-R (*A*) or Na gluconate-R (*B*) and treated with amiloride and ouabain as in Fig. 9. This was followed by (*A*) replacement of serosal NaCl-R with NMDG Cl-R containing 7.5 mM Na, or (*B*) replacement of serosal Na gluconate-R with NMDG gluconate-R containing 7.5 mM Na

trode voltage difference ($V_K - V_o$) corresponding to a_K^c. Under control conditions a_K^c was 95 mM. Exposure to serosal Na_2SO_4-R caused partial progressive depolarization of V_o for about 40 min, in association with a decline of a_K^c to a steady-state value of 55 mM. The response to amiloride validated the impalements, with marked hyperpolarization of V_o and, after a short transient, insignificant change of $V_K - V_o$ (García-Díaz et al., 1985). Attempts to observe recovery of a_K^c were abandoned, since we were unable to maintain two impalements simultaneously for more than 90 min. The results of five experiments similar to that of Fig. 11 are summarized in Table 1. Although a_K^c decreased in every tissue, E_K fell on the average only 7 mV, due to the concomitant decrease of serosal K activity from 2.0

Table 1. Effects of serosal SO_4 and gluconate on a_K^c

Skin #	V_o (mV)	a_K^c (mM)	E_K (mV)	V_o (mV)	a_K^c (mM)	E_K (mV)
A	NaCl-R			Na_2SO_4-R		
1	−58	115	107	−30	57	97
2	−82	86	99	−54	63	99
3	−78	117	107	−23	43	90
4	−65	94	101	−41	60	98
5	−83	109	105	−48	67	101
Mean ± SEM	−73 ± 5	104 ± 6	104 ± 2	−39 ± 6	58 ± 4	97 ± 2
B	NaCl-R			Na gluconate-R		
6	−18	70	93	−19	102	103
7	−30	108	104	−19	136	110
8	−51	87	99	−29	102	103
Mean ± SEM	−33 ± 10	88 ± 11	98 ± 3	−22 ± 3	113 ± 11	105 ± 2

Measurements were made before replacement of serosal NaCl-R, (*A*) after 40–50 min in serosal Na_2SO_4-R, and (*B*) after 22, 77, and 28 min in Na gluconate-R. The activity coefficient of K in Na gluconate-R was taken as 0.78.[2]

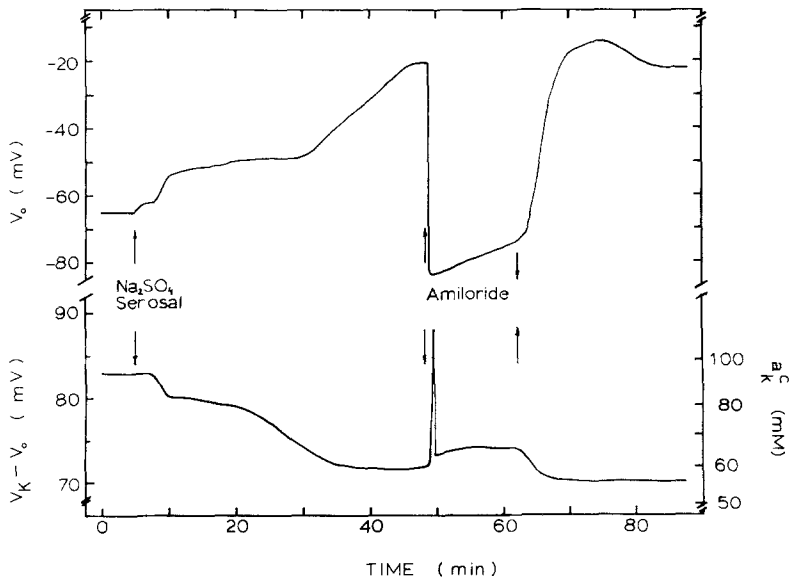

Fig. 11. Decrease of a_K^c due to replacement of serosal Cl with SO_4. The short-circuited skin was first equilibrated with serosal NaCl-R. After recording baseline values the serosal perfusate was changed to Na_2SO_4-R. The upper trace shows the response of V_o. The lower trace shows the difference between V_K and V_o, which corresponds to a_K^c. (The scale on the right was derived by calibration of the K electrode used in this experiment.) After a 40-min period in Na_2SO_4-R, amiloride was administered to verify the validity of the measurements

to 1.3 mM. So slight a change in the K equilibrium potential cannot account for the degree of depolarization observed.

It was also of interest to examine the effect on a_K^c of substitution of gluconate for Cl, particularly in view of a report of electron microprobe analysis showing an increase of cell K concentration under these conditions (Dörge et al., 1985). Our findings agreed. Results of a representative experiment are shown in Fig. 12. In three cases, following an initial slight decrease, a_K^c rose to a steady-state value well above the control level (Table 1*B*); E_K rose slightly.[3]

[2] The activity coefficient of Na in 0.05 M Na_2SO_4 solution was found to be 0.58 and 0.61, as measured with two different calibrated Na-selective electrodes. The values of E_K calculated when γ_K is considered to be 0.60 rather than 0.52 differ by 3.7 mV from the values of Table 1.

[3] Our results are on the other hand apparently inconsistent with the finding by electron microprobe analysis that the intracellular K concentration was virtually unchanged in 5 skins of *Rana temporaria* after 40–60 min of replacement of medium Cl by SO_4 (R. Rick, *personal communication*). In that study, however, the SO_4-R was adjusted to an osmolality of 200 mOsm/Kg H_2O in order to minimize changes in cell volume and short-circuit current. Also to be considered are possible effects of SO_4-R on intracellular activity coefficients.

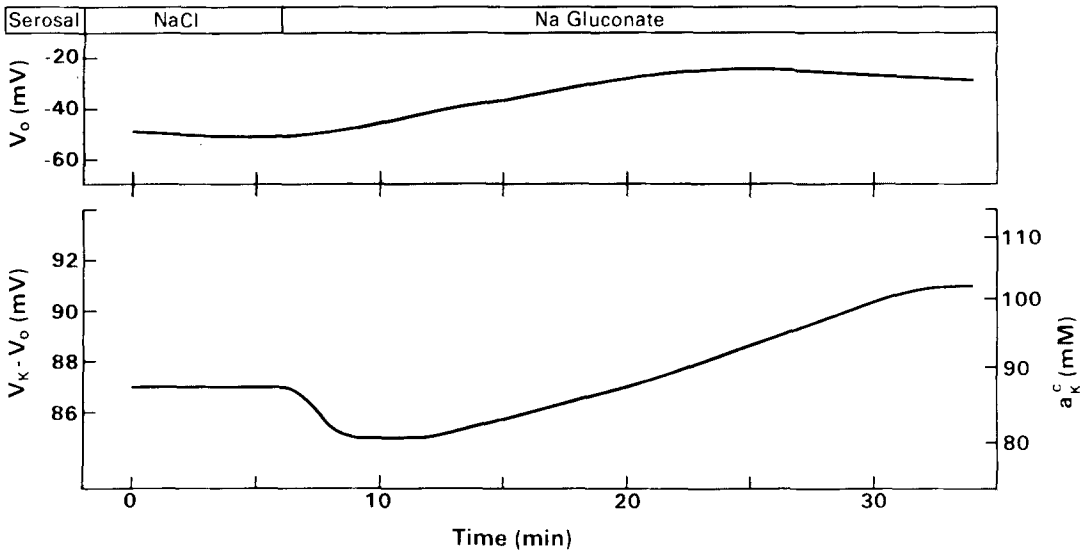

Fig. 12. Increase of a_K^c due to replacement of serosal Cl with gluconate. After equilibration of the short-circuited skin with NaCl-R, the serosal solution was replaced with Na gluconate-R. Traces of V_o and $V_K - V_o$ are presented as in Fig. 11

Table 2. Effects of Serosal SO_4 on a_{Na}^c

Skin #	NaCl-R				Na_2SO_4-R			
	I_c (μA)	V_o (mV)	a_{Na}^c (mM)	$E_{Na,o}$ (mV)	I_c (μA)	V_o (mV)	a_{Na}^c (mM)	$E_{Na,o}$ (mV)
1	86.1	−78	11.5	52.4	61.1	+13	1.2	111.3
2	45.8	−58	13.5	48.2	18.1	−20	0.9	118.8
3	63.9	−40	8.7	59.6	43.1	−12	1.2	111.3
Mean	65	−59	11	53	41	−6	1.1	114
SEM	12	11	1	3	13	10	0.1	3

Measurements were made before and 30–40 min after replacement of serosal NaCl-R by Na_2SO_4-R. $E_{Na,o}$, the Nernst potential across the outer membrane, was calculated from the measured a_{Na}^c and a_{Na} of the mucosal bathing solution. In three additional skins, data were obtained which do not fully satisfy our standard criteria in that impalements with both electrodes were not maintained continuously throughout the interval between reported observations; these data were similar to those shown.

Because of the marked decrease of a_K^c following exposure to serosal SO_4, we also examined the effect on cell Na activity. As is shown in Table 2, there was a marked decrease. This supports our conclusion that the effects of serosal SO_4 are not attributable to inhibition of the basolateral Na pump.

Discussion

Several groups have examined diverse aspects of Cl transport in the frog skin by removing Cl from one or both baths. Biber and his colleagues (1985) have made comprehensive studies of skins of Southern *Rana pipiens* and have concluded from unimpressive initial electrical effects following bilateral Cl removal that Cl transport is primarily electroneutral. Cell Cl activity measurements demonstrated apical and basolateral Cl transport via Na-transporting principal cells and implicated an apical Cl-HCO_3 exchange mechanism (Biber et al., 1985; Drewnowska & Biber, 1988). Several studies have emphasized basolateral cotransport, varying with different preparations and conditions. Duffey et al. (1986) found that serosal Cl substitution induced cell alkalinization, consistent with basolateral Cl-HCO_3 antiport. Other substitution experiments have pointed to basolateral NaCl cotransport (Ferreira & Ferreira, 1981; Giraldez & Ferreira, 1984; Dörge et al., 1985), whereas studies of volume regulation have suggested basolateral $NaKCl_2$ cotransport (Ussing, 1985). On the other hand, isotope kinetic studies of Stoddard, Jakobsson and Helman (1985) have indicated electroneutral basolateral Cl transport, for the most part uncoupled to transport of either Na or K. Cellular electrical effects of serosal Cl removal have been described by Biber et al. (1985), by Duffy et al. (1986), and by Leibowich, DeLong and Civan (1988). Our findings provide additional information concerning membrane electrophysiology and cell cation activities.

Because of effects on activity coefficients, the substitution of SO_4 for Cl reduces cation activities in the serosal bath. On the other hand, activity coefficients are not affected by monovalent anions, ex-

cept for Ca in gluconate solution (Christoffersen & Skibsted, 1975). The qualitative similarity of the effects of serosal SO_4, gluconate, and NO_3 on V_o and g_i suggests that these effects may be due to the removal of Cl ions *per se*.

As depolarization of V_o on Cl replacement is not attributable either to inhibition of the Na pump or to lowering of E_K, we presume that it is due to diminution of basolateral conductance. In all the experiments of Figs. 4 and 5, g_i decreased. It is appreciated that values of the slope conductance g_i are only estimates of the electrical conductance of the inner membrane, since as shown elsewhere (Nagel, 1985; Klemperer et al., 1986*b*) different formulations yield different values. We have chosen the method of Frömter and Gebler (1977), because it takes account of incomplete inhibition of Na transport, hence permitting exposure to amiloride to be brief, which facilitates reversibility. However, the basolateral chord conductance G_i of an alternative method (Nagel, 1985) (*see* Table of Main Symbols Used) showed a qualitatively similar response to that of g_i; following Cl replacement by SO_4, g_i fell from 2.3 ± 0.5 to 0.6 ± 0.2 mS/cm^2 while G_i dropped from 0.94 ± 0.11 to 0.32 ± 0.04 mS/cm^2 (n = 7). A similar result was found for Cl substitution by gluconate or NO_3.

Because basolateral conductance is mainly attributable to K, large changes of g_i and G_i suggest change of g_K. With imperfect selectivity, however, it is necessary to consider also other conductances. We examined those of Cl and Na. With serosal SO_4, these were not important. Enhancement of basolateral Cl conductance in association with depolarization, as described by Ussing (1982), did not occur after replacement of serosal Cl with SO_4 (Fig. 8*B*). Prolonged serosal exposure to sulfate appeared to increase an initially small Na conductance, but the effect was slight. Although diminished depolarization by high $[K]_s$ (Fig. 7) might in principle be due to enhanced conductance of other species, the concurrent fall in g_i indicates a decline of g_K as well. The same consideration applies with serosal gluconate, despite modest effects on both Cl conductance (Fig. 9*B*) and Na conductance (Fig. 10). (Here it is of interest that Lewis et al. (1985) observed that replacement of serosal Cl with gluconate reduced the sensitivity of Na transport to serosal Ba in the toad urinary bladder, suggesting a decrease of basolateral K conductance.)

Our findings raise questions concerning mechanisms, which we are presently unable to resolve. For one, although loss of cell K in cotransport with Cl would account for the fall of a_K^c found with serosal SO_4-R, it cannot account for the lowering of g_i, because this was seen also with gluconate-R, which has been found to raise a_K^c (Table 1*B*) and the mean cell K concentration (Dörge et al., 1985).

Another significant question is how serosal Cl replacement influences cell current. With serosal gluconate, studied by us in whole skins, effects were insignificant or minimal, despite marked cell depolarization. Leibowich et al. (1988) have reported similar findings in whole skins; in isolated epithelia, on the other hand, serosal gluconate regularly inhibited the short-circuit current. On the basis of analysis of the apical amiloride-sensitive current-voltage relationship, it was concluded that serosal gluconate does not inhibit either apical Na permeability or the basolateral Na pump, but likely reduces cell volume, triggering inhibition of basolateral K channels. This conclusion is consistent with the findings of Lewis et al. (1985) and with those of the present study.

With serosal SO_4, we found I_c to be depressed consistently. At the apical membrane, although $E_{Na,o}$ and V_o were altered greatly, the electrochemical potential difference for Na was unaffected; $(E_{Na,o} - V_o)$ was 112 ± 9 mV before Cl substitution and 120 ± 12 mV after Cl substitution (Table 2). These findings underscore the fact that thermodynamically equivalent values of $E_{Na,o}$ and V_o cannot be assumed to have equivalent effects on transport except in the immediate vicinity of equilibrium. In keeping with this consideration, the chord conductance $(G_o \equiv I_c/(E_{Na,o} - V_o))$ decreased, although the slope conductance g_o was unchanged (Figs. 4 and 5). Lowering of a_{Na}^c will of course reduce basolateral Na transport, but it is noteworthy that, with serosal SO_4-R, I_c averaged about two-thirds of control value, despite a_{Na}^c levels lower than found on abolition of net transport by the administration of amiloride or removal of mucosal Na (García-Díaz et al., 1986). Possibly this difference is attributable to the magnitude of the positive electrical potential step at the basolateral surface. In the study of Table 2, the serosal substitution of SO_4 for Cl lowered V_i to 6 ± 10 mV, whereas in the study of García-Díaz et al. (1986); the value of V_i was 88 ± 5 (SD) mV following the administration of amiloride and 109 ± 9 (SD) mV following the removal of mucosal Na.

Finally, the large decrease of both a_K^c and a_{Na}^c induced by serosal SO_4-R raises the question of how cell osmolality is maintained (Macknight & Leaf, 1977; Kregenow, 1981). Pertinent studies in epithelia are lacking.

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References

Biber, T.U.L., Drewnowska, K., Baumgarten, C.M., Fisher, R.S. 1985. Intracellular Cl activity changes of frog skin. *Am. J. Physiol.* **249:**F432–F438

Candia, O.A., Reinach, P.S. 1977. Sodium washout kinetics across inner and outer barriers of the isolated frog skin epithelium. *Biochim. Biophys. Acta* **468:**341–352

Christoffersen, G.R.J., Skibsted, L.H. 1975. Calcium ion activity in physiological salt solutions: Influence of anions substituted for chloride. *Comp. Biochem. Physiol.* **52:**317–322

Cox, T.C., Helman, S.I. 1983. Effects of ouabain and furosemide on basolateral membrane Na efflux of frog skin. *Am. J. Physiol.* **245:**F312–F321

DeLong, J., Civan, M.M. 1984. Apical sodium entry in split frog skin: Current-voltage relationship. *J. Membrane Biol.* **82:**25–40.

Dörge, A., Rick, R., Beck, F., Thurau, K. 1985. Cl transport across the basolateral membrane in frog skin epithelium. *Pfluegers Arch.* **405 (Suppl. 1):**S8–S11

Drewnowska, K., Biber, T.U.L. 1988. Effect of changes in extracellular Cl on intracellular Cl activity in frog skin. *Am. J. Physiol.* **254:**F95–F104

Duffey, M.E., Kelepouris, E., Peterson-Yantorno, K., Civan, M.M. 1986. Microelectrode study of intracellular pH in frog skin: Dependence on serosal chloride. *Am. J. Physiol.* **251:**F468–F474

Ferreira, K.T.G., Ferreira, H.G. 1981. The regulation of volume and ion composition in frog skin. *Biochim. Biophys. Acta* **646:**193–202

Frömter, E., Gebler, B. 1977. Electrical properties of amphibian urinary bladder epithelia: III. The cell membrane resistance and the effect of amiloride. *Pfluegers Arch.* **371:**99–108

García-Díaz, J.F., Baxendale, L.M., Klemperer, G., Essig, A. 1985. Cell K activity in frog skin in the presence and absence of cell current. *J. Membrane Biol.* **85:**143–158

García-Díaz, J.F., Klemperer, G., Baxendale, L.M., Essig, A. 1986. Cell sodium activity and sodium pump function in frog skin. *J. Membrane Biol.* **92:**37–46

Giraldez, F., Ferreira, K.T.G. 1984. Intracellular chloride activity and membrane potential in stripped frog skin (*Rana temporaria*) *Biochim. Biophys. Acta* **769:**625–628

Klemperer, G., García-Díaz, J.F., Essig, A. 1986*a*. Decreased K conductance at the basolateral membrane in frog skin bathed in serosal Cl free solutions. The XXX Congress of the International Union of Physiological Sciences, Vancouver, Canada

Klemperer, G., García-Díaz, J.F., Nagel, W., Essig, A. 1986*b*. Basolateral membrane potential and conductance in frog skin exposed to high serosal potassium. *J. Membrane Biol.* **90:**89–96

Kregenow, F.M. 1981. Osmoregulatory salt transporting mechanisms: Control of cell volume in anisotonic media. *Annu. Rev. Physiol.* **43:**493–505

Leibowich, S., DeLong, J., Civan, M.M. 1988. Apical Na^+ permeability of frog skin during seasonal Cl^- replacement. *J. Membrane Biol.* **102:**121–130

Lewis, S.A., Butt, A.G., Bowler, M.J., Leader, J.P., McKnight, A.D.C. 1985. Effects of anions on cellular volume and transepithelial Na^+ transport across toad urinary bladder. *J. Membrane Biol.* **83:**119–137

Macknight, A.D.C., Leaf, A. 1977. Regulation of cellular volume. *Physiol. Rev.* **57:**510–573

Nagel, W. 1985. Basolateral membrane ionic conductance in frog skin. *Pfluegers Arch.* **405 (Suppl. 1):** S39–S43

Nagel, W., García-Díaz, J.F., Essig, A. 1983. Contribution of junctional conductance to the cellular voltage-divider ratio in frog skins. *Pfluegers Arch.* **399:**336–341

Nagel, W., García-Díaz, J.F., Essig, A. 1988. Voltage dependence of cellular current and conductances in frog skin. *J. Membrane Biol.* **106:**13–28

Stoddard, J.S., Jakobsson, E., Helman, S.I. 1985. Basolateral membrane chloride transport in isolated epithelia of frog skin. *Am. J. Physiol.* **249:**C318–C329

Ussing, H.H. 1982. Volume regulation of frog skin epithelium. *Acta Physiol. Scand.* **114:**363–369

Ussing, H.H. 1985. Volume regulation and basolateral co-transport of sodium, potassium, and chloride ions in frog skin epithelium. *Pfluegers Arch.* **405 (Suppl. 1):** 2–7

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