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Microelectrode Study of K^+ Accumulation by Tight Epithelia: II. Effect of Inhibiting Transepithelial Na^+ Transport on Reaccumulation following Depletion

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Summary. The effects of restoring serosal potassium to potassium-depleted toad urinary bladders have been re-examined using double-barrelled microelectrodes. The data confirm the existence of a time-lag phenomenon, a dissociation between potassium reaccumulation and restoration of short-circuit current. Returning serosal potassium stimulates an increase in intracellular potassium activity 21–26 min before any increase can be detected in short-circuit current.

The reaccumulation of potassium has been further studied using split frog skin, a far more suitable preparation for electrophysiologic study than toad bladder. Under baseline short-circuited conditions, potassium is accumulated against an electrochemical gradient of 22 ± 4 mV. Reaccumulation of potassium by potassium-depleted tissues can be blocked by inhibiting the Na,K-exchange pump with high concentrations of ouabain. On the other hand, blocking apical sodium entry by the addition of 10^{-4} M amiloride to the outer bathing medium does not interfere with reaccumulation of potassium.

The data support the concept that the time-lag phenomenon of toad bladder reflects stimulation of potassium reaccumulation by the sodium pump in exchange for the extrusion of excess cell sodium collected during the period of potassium depletion. This reaccumulation of potassium can proceed before the entry of significant added amounts of sodium across the apical plasma membrane.

Key Words time-lag phenomenon · amiloride · ouabain · split frog skin · toad urinary bladder · Na,K-exchange pump · apical Na^+ entry

Introduction

A number of published data have suggested that potassium accumulation need not necessarily be linked to net sodium transport across epithelia (Essig & Leaf, 1963; Curran & Cereijido, 1965; Essig, 1965; Biber, Aceves & Mandel, 1972; Candia & Zadunaisky, 1972; Giebisch, Sullivan & Whittembury, 1973; Nellans & Schultz, 1976; Robinson & Macknight, 1976*c*; Kimura & Fujimoto, 1977; Kimura et al., 1977; Valenzeno & Hoshiko, 1977; DeLong & Civan, 1978). A particularly striking example of such dissociation has been provided by monitoring intracellular K^+ activity (a_K^c) and short-circuit current (I_{sc}) of toad urinary bladder during the course of transiently reducing the serosal K^+ concentration (c_K^s) (Delong & Civan, 1978). [The short-circuit current is the electrical equivalent of net Na^+ transport across frog skin (Ussing, 1960) and toad bladder (Leaf, 1965) under many experimental conditions.] After initially stimulating the short-circuit current, reduction in c_K^s decreased intracellular K^+ activity, short-circuit current, and transepithelial electrical conductance (G_T). These changes were reversed upon restoring K^+ to the serosal medium, but with distinctly different time courses. Restoration of serosal K^+ produced increases in a_K^c, I_{sc} and G_T. However, the increases in short-circuit current and conductance lagged that in K^+ activity by 20–40 min; we refer to this observation as the time-lag phenomenon.

The physiological basis for this phenomenon has been far from clear. One possibility is that the effect may have, in some way, reflected impalement-induced membrane damage. This explanation is supported by recent documentation of impalement-induced damage in toad bladder (DeLong & Civan, 1983). With varying degrees of membrane damage, calculation of a_K^c from separate impalements with an ion-selective microelectrode and reference micropipette could be inappropriate. For this reason, a_K^c and I_{sc} have been remonitored in toad bladder with double-barrelled microelectrodes during the course of transiently reducing c_K^s. These results have been presented in preliminary form (DeLong & Civan, 1979).

An alternative interpretation of the time-lag phenomenon is that the effect arises from stimulation of $Na^+ - K^+$ exchange through the basolateral pump before restoration of c_K^s has produced any increase in apical Na^+ entry into the transporting cells from the mucosal medium. This explanation is based upon the presumption that the transient reduction in serosal K^+ produces not only a loss

in intracellular K^+ conductance, but also a substantial gain in cell Na^+. This assumption has been supported by electron probe X-ray microanalysis of toad bladder (Civan, Hall & Gupta, 1980). The underlying presumption is examined in further detail in the present report.

Although the initial experiments of the current study were conducted with the urinary bladder of the toad, most of the data have been obtained with frog skins. As documented elsewhere, frog skin is a far more suitable preparation than toad bladder for the electrometric measurement of a_K^c (DeLong & Civan, 1983). Split, rather than whole, frog skins have been studied, in order to enhance the rate of exchange between intracellular and serosal potassium (Fisher, Erlij & Helman, 1980). The electrical properties of split and whole frog skins have been reported to be otherwise similar (Aceves & Erlij, 1971; Fisher, Erlij & Helman, 1980).

Materials and Methods

The experimental approach has been presented in another paper (DeLong & Civan, 1983), which should be consulted for further information.

Urinary hemibladders from doubly-pithed toads (*Bufo marinus*) were mounted mucosal side up between the two halves of a Lucite chamber (DeLong & Civan, 1978). Skins from doubly-pithed frogs (*Rana pipiens pipiens*) were mounted inner side up in a similar chamber. However, before mounting, the underlying corium was removed from the skin by the enzymatic method of Fisher et al. (1980). A tissue area of 1.9 cm^2 was available for experimental study.

Several bathing media were used in the present study (Table 1). The standard Ringer's solution (R) contained 3.5 mM K^+; the osmolality was 215–225 mOsm, and the pH 7.6–7.8. Ringer's solutions nominally free of potassium (0K) and containing 80.0 mM potassium (80K) were also used. In addition, a number of solutions containing low concentrations of K^+ were prepared by mixing volumes of 0K and R Ringer's solutions in suitable proportion. The mixture most commonly applied to the tissues of the current work was a medium containing 0.85 mM potassium (0.85K). The pH and osmolality of all media were similar to those of the standard Ringer's solution.

Toad bladders were impaled with double-barrelled microelectrodes constructed from theta glass tubing. Frog skins were studied with single-barrelled microelectrodes drawn from omega-dot capillary glass tubing. The reference sides of the double-barrelled microelectrodes were filled with 1 M magnesium acetate solution in order to suppress the dependence of these fine-tipped micropipettes on the ionic composition of the test media (Acker, 1978; DeLong & Civan, 1979); when filled with 3 M KCl solution and tested in Ringer's solution, such reference barrels had resistances as high as 100 MΩ. The single-barrelled micropipettes had lower resistance (approximately 40 MΩ under similar conditions), and were insensitive to the ionic strength of the ambient medium; such micropipettes were filled with 0.5 M KCl solution in order to reduce the rate of release of KCl into the cell during extended impalements (Nelson, Ehrenfeld & Lindemann, 1978). Criteria for acceptability of impalements have been presented elsewhere (DeLong & Civan, 1983) and are discussed further in the Results.

Table 1. Compositions of Ringer's solutions (in mM)

Component	R	0 K	80 K
Na^+	115.1	118.6	35.0
K^+	3.5	0	80.0
Ca^{2+}	0.8	0.8	0.8
Cl^-	113.9	113.9	110.5
HCO_3^-	2.2	2.2	2.2
HPO_4^{2-}	1.8	1.8	1.8
$H_2PO_4^-$	0.3	0.3	0.3
Sucrose	0	0	0

The values of membrane potential reported in the current manuscript and used in the calculation of intracellular potassium activity have been corrected for junction potentials at the tips of the reference micropipettes (DeLong & Civan, 1983). The total correction amounted to making the membrane potentials for toad bladder 5.7 mV more negative, and those for split frog skin 7.3 mV more negative than the values directly measured.

The intracellular potassium activity (a_K^c) was calculated from the equation (DeLong & Civan, 1978):

$$a_K^c = (a_K + k a_{Na})_e 10^{(\Delta E_K^c - \Delta\psi_c)/S} \quad (1)$$

where k and S are selectivity and sensitivity constants, respectively, of the microelectrode, a_{Na} is the sodium activity, and c and e refer to the cellular and extracellular phases, respectively. ΔE_K^c and $\Delta\psi_c$ are the intracellular voltages measured with the potassium-selective microelectrode and reference micropipette, respectively, with reference to the extracellular values. The mean values ±SEM of k and s were 0.020 ± 0.002 and 59.5 ± 0.5 mV, respectively.

The transmural potential ($\Delta\psi_T$) was measured and clamped at 0 mV except for periodic hyperpolarizations of 10–20 mV for 3–10 sec (Civan & Hoffman, 1971). The transepithelial current (I_T) and the outputs of the microelectrode (E_K) and reference (ψ) barrels were continuously monitored. The difference ($E_K - \psi$) was often displayed, as well.

Unless otherwise stated, values are presented as the mean ±SEM, with the number of measurements entered in parentheses.

Results

Toad Bladder

Several lines of evidence have suggested that impalements of the urinary bladder of the toad are commonly associated with significant leakage around the micropipette tip (Lindemann, 1975; Higgins, Gebler & Frömter, 1977; Sudou & Hoshi, 1977; DeLong & Civan, 1979, 1980, 1983). To deal with this problem, a null point technique already described in detail (DeLong & Civan, 1979, 1980, 1983) has been applied. The basic strategy is to superfuse the mucosal surface with Ringer's solutions containing high concentrations of potassium (80K, Table 1); this minimizes the difference in electrochemical potential for K^+ across the apical membrane, and thereby minimizes the redistribu-

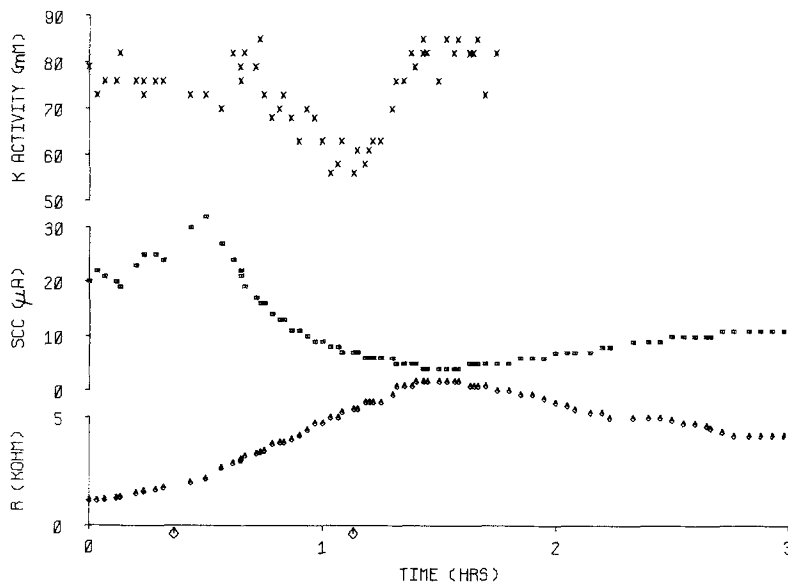


Fig. 1. Impalement of short-circuited toad bladder with double-barrelled microelectrode. The reference barrel was filled with 1 M Mg acetate. Intracellular K^+ activity (a_K^c), short-circuit current (SCC), and transmural resistance (R) are displayed as functions of time. In contrast to the succeeding figures obtained with split frog skin, each value of a_K^c was based on impalement of a different cell. During the period bracketed by the rhomboids, the tissue was bathed with nominally potassium-free serosal medium

tion of potassium across that membrane, under baseline conditions even in the presence of membrane damage.

Figure 1 illustrates the results obtained with fine-tipped double-barrelled microelectrodes in two successful experiments with toad bladder. Each value of a_K^c is based upon a separate impalement in a different cell. After transiently stimulating short-circuit current (Essig, 1965; Finn, Handler & Orloff, 1967; Robinson & Macknight, 1976*a*, *b*), removal of potassium from the serosal bath both reduced a_K^c and short-circuit current and increased tissue resistance. Restoration of serosal potassium reversed these changes. However, intracellular potassium activity began to increase well before short-circuit current and tissue resistance began to return towards their baseline values. The time-lag noted in the two experiments was 21 and 26 min.

It will be appreciated that with the reduction of a_K^c by lowering serosal K^+ concentration, the high mucosal K^+ (c_K^m) concentration of 80 mM no longer minimizes the redistribution of K^+ across the apical membrane. Rather, impalement-induced damage would permit influx of K^+, factitiously increasing the measured value of a_K^c. To estimate the true absolute value of a_K^c in the steady-state, we would have to progressively lower the mucosal K^+ concentration in order to obtain the new null point under the altered experimental conditions. In the present study, we are not concerned with the absolute value of a_K^c, but rather in the qualitative demonstration of the time-lag phenomenon. The current results document that the phenomenon can be observed not only when a_K^c is likely to be underestimated ($c_K^m = 3.5$ mM; DeLong & Civan, 1978), but when a_K^c is likely to be overestimated ($c_K^m = 80$ mM). However, the principal value of this part of the study was to study a_K^c with double-barrelled microelectrodes, where measurements of ΔE_K^c and $\Delta \psi_c$ are directly comparable.

Split Frog Skin

As noted in the Introduction, the bulk of the present work was carried out with split frog skin, rather than with toad bladder because of the far greater ease in obtaining satisfactory impalements with the latter tissue. Although we have been able to obtain stable impalements in toad bladder for 20–30 sec, stable intracellular recordings can be obtained with whole and split skins for periods sometimes as long as hours. Despite this, it would be misleading to dimiss the technical difficulties in the present study. The most direct approach for measuring intracellular potassium activity would be to impale the epithelial cells with double-barrelled microelectrodes. Although this approach is feasible, the success rate in fabricating suitable microelectrodes is too low to be practicable (DeLong & Civan, 1983). Instead, we have elected to obtain simultaneous intracellular recordings from an ion-selective microelectrode and reference micropipette placed in two different cells. This approach is based on the observation that the epithelium of frog skin constitutes a functional syncytium (Nagel, 1976; Rick, Dörge, von Arnim & Thurau, 1978*b*; DeLong & Civan, 1983). Because the experimental perturbations of the present study can produce complex changes in intracellular potential with time, sepa-

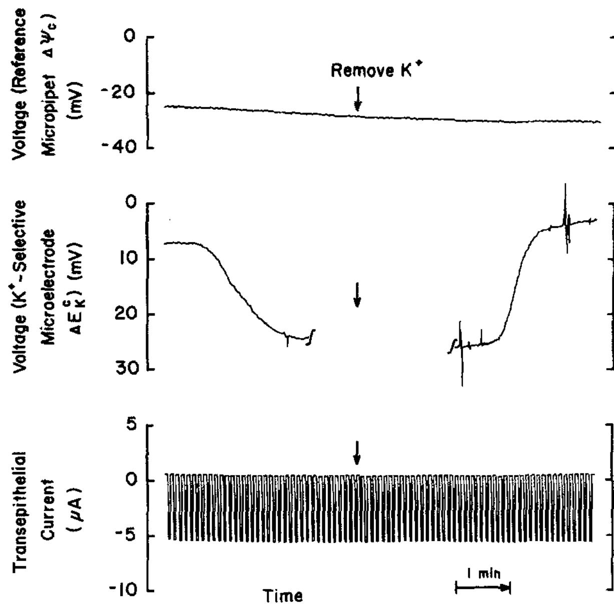


Fig. 2. Response of extracellularly placed potassium-selective microelectrode following restoration of potassium to the inner bathing medium. The reference micropipette and ion-selective microelectrode had both been introduced into epithelial cells. During the course of the experiment, the microelectrode appeared to slip out of place, and was therefore withdrawn into the extracellular space. The reference micropipette remained in position. The inner bathing medium was then changed to a Ringer's solution containing 0.85 mM potassium in preparation for the next impalement. The response of the potassium-selective microelectrode within the interstitial space was similar to that noted during the calibration procedure in simple aqueous solutions. The magnitude and time course of the response to the change in bathing solutions was characteristic of microelectrodes placed in the extracellular space and provided an additional criterion for determining the placement of the microelectrode tip

rate impalements with a potassium-selective microelectrode and reference micropipette would be inadequate for the present purposes.

An additional technical problem was the reduced number of criteria which could be used for determining the acceptability of any given impalement. As usual, minimal criteria for acceptability included: (i) rapid changes in potential upon entering and leaving a cell, (ii) constancy of the potentials measured in the extracellular fluid before and after penetration, usually to within 3 mV, and (iii) constancy of the value of S to within 3 mV when measured before and after impalements. However, additional criteria which can be used under baseline conditions were not necessarily helpful in the present work. In particular, transient applications of amiloride, and of barium or strophanthidin in order to produce brief hyperpolarizations and depolarizations, respectively (DeLong & Civan, 1983), were usually inapplicable; either

Table 2. Dependence of short-circuit current (I_{sc}) upon potassium concentration of medium bathing inner surface of split frog skin

c^s_K (mM)	Reduction in I_{sc} (%)		Half-time for change in I_{sc} (min)			
			Decay		Recovery	
	Measurements	Mean	Measurements	Mean	Measurements	Mean
3.5	–	0	–	–	–	–
2.5	3	−2	–	–	–	–
	−7					
0.85	36	37	12	14	16	15
	32		14		17	
	35		16		–	
	44		13		11	
0.35	72	72	24	24	9	9
0.18	72	72	18	18	16	16
0.0	73	76	21	18	10	10
	79		16		9	

The half-times for decay and recovery include a delay time of 1.3–2 min before the superfusing solution reached the tissue

the response to the agent was altered by the operating conditions, or introduction of the agent might have altered the subsequent response to restoration of potassium. This was of particular concern for impalements with the potassium-selective microelectrodes across the basolateral membrane; the measured values of ΔE^c_K are not very different from zero (DeLong & Civan, 1983), and the fractional resistance across the basolateral membrane is characteristically much smaller than across the apical membrane. Under these circumstances, the additional criterion illustrated by Fig. 2 proved helpful. When the tip of the ion-selective microelectrode was placed in the interstitial space, a characteristic response to changes in potassium concentration of the inner medium could be observed. The magnitude of the response could be predicted from the calibration of the microelectrode in simple standard solutions; the time course of the response was also usually faster than that observed for an intracellular record.

One final experimental difficulty was encountered in preliminary measurements of a^c_K during the course of removing serosal potassium. For reasons unclear, impalements appeared to be appreciably less stable in potassium-free Ringer's solution than in standard Ringer's solution. This problem was solved by reducing the potassium concentration of the inner medium not to 0, but to some intermediate value. In order to choose a suitable intermediate value for c^s_K, the short-circuit currents

Table 3. Intracellular chemical and electrochemical activities of potassium and basolateral membrane potential during transient reductions in potassium concentration of inner bathing medium

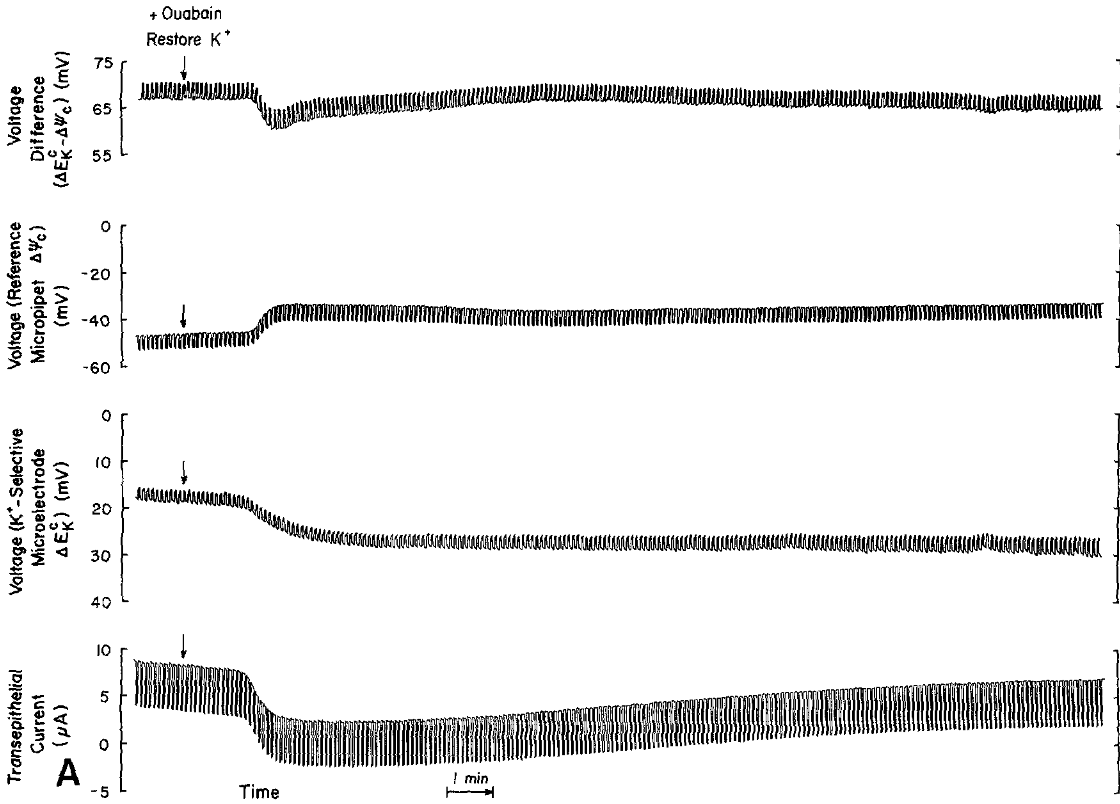
Preparation	Run	Baseline/fully recovered				Depleted				Partially recovered/less depleted			
		a_K^c (mM)	$\Delta\psi_c$ (mV)	a_K^s (mM)	$\Delta\tilde{\mu}_K^{bl}$ (mV)	a_K^c (mM)	$\Delta\psi_c$ (mV)	a_K^s (mM)	$\Delta\tilde{\mu}_K^{bl}$ (mV)	a_K^c (mM)	$\Delta\psi_c$ (mV)	a_K^s (mM)	$\Delta\tilde{\mu}_K^{bl}$ (mV)
I	A	100	−92	2.66	0	38	−69	0	–	–	–	–	–
	B	103	−91	2.66	2	13	−41	0	–	53	−85	2.66	−9
II	A	91	−57	2.66	33	–	–	–	–	–	–	–	–
	B	105	−63	2.66	30	74	−60	0.65	60	84	−62	2.66	26
	C	–	–	–	–	46	−41	0.27	90	61	−59	0.65	56
	D	–	–	–	–	44	−45	0.65	62	69	−58	2.66	25
III	A	111	−73	2.66	22	52	−73	0.65	38	–	–	–	–
	B	104	−63	2.66	30	65	−66	0.65	51	–	–	–	–
	C	107	−66	2.66	28	58	−63	0.65	51	–	–	–	–
	D	112	−71	2.66	24	69	−67	0.65	51	–	–	–	–
	E	–	–	–	–	74	−54	0.65	66	91	−63	2.66	27
IV	A	101	−59	2.66	33	81	−59	0.65	64	–	–	–	–
	B	103	−73	2.66	20	71	−62	0.65	57	–	–	–	–

across two split skins were monitored during the course of transiently reducing the serosal potassium concentration to a series of values. The percentage inhibitions and half-times for decay and recovery are presented in Table 2. A serosal potassium concentration of 0.85 mM proved to be optimal. As indicated in the Table, the degree of inhibition was substantial, half that obtained with nominally potassium-free medium, and impalements could be performed as easily as under baseline conditions. For these reasons, most of the potassium depletions were carried out by lowering c_K^s to 0.85 mM.

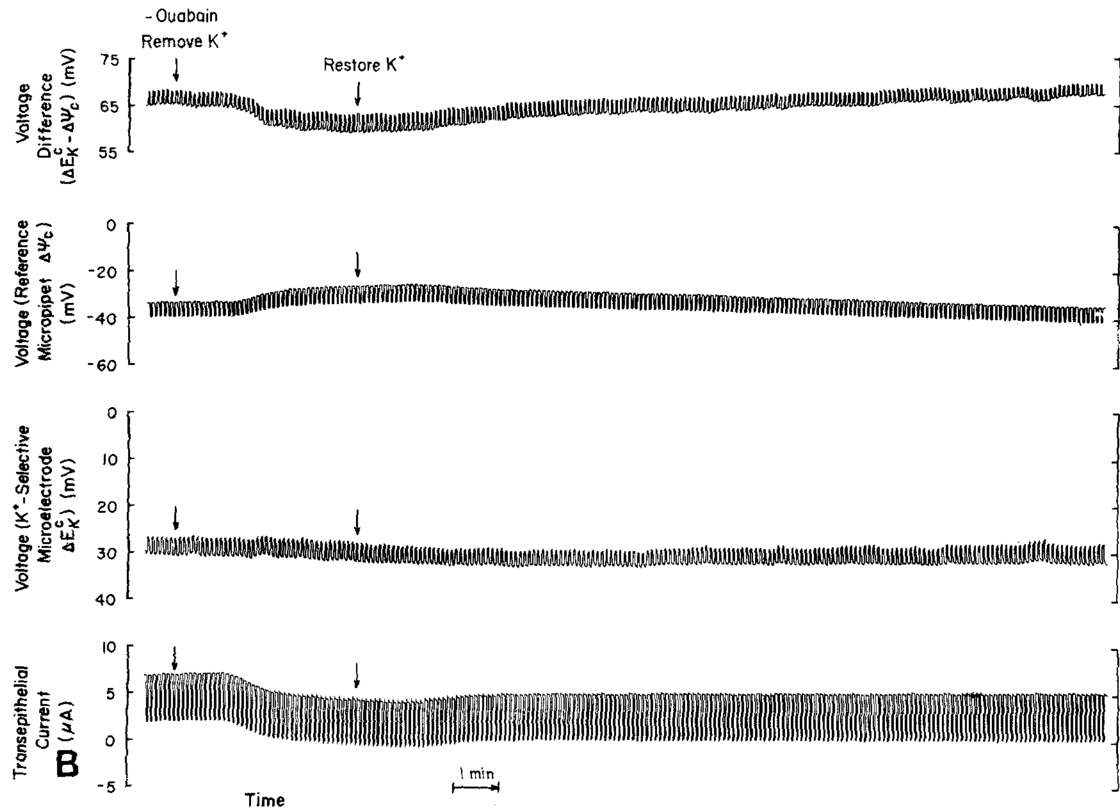
Table 3 summarizes the results obtained with four split frog skins under baseline conditions, after transiently depleting the tissues of potassium and after partial or complete restoration of intracellular potassium. The experimental runs refer to separate comparisons of a_K^c in the same tissue bathed with different serosal potassium concentrations. Bathed with a serosal potassium concentration of 3.5 mM under short-circuited conditions, the intracellular potassium activity was measured to be 91–112 mM. The first control entry of Table 3 (IA) had actually been included in a series of baseline values in a preceding manuscript (DeLong & Civan, 1983). Excluding this value, the mean ±SEM is calculated to be 104±2 mM, identical to the value previously measured of 104±3 mM. The Table also indicates that transiently reducing c_K^s from 3.5 to 0.85 mM (corresponding to a reduction in serosal potassium activity from 2.66 to 0.65 mM) reduced intracellular potassium activity to 44–81 mM (mean ±SEM=65±4 mM). The wide range in reductions reflects the variable periods of depletion (mean±SEM=10±2 min). As noted in the Table, reducing c_K^s still further led to falls in a_K^c to as low as 13 mM. These reductions were partially or completely reversible over periods of repletion lasting 13±2 min. The headings *Partially recovered* and *Fully recovered* refer solely to the intracellular K^+ activity, and not to the short-circuit current.

Although not indicated in Table 3, the half-time for the reduction of a_K^c following the lowering of serosal K^+ concentration varied from about 3.5 to 15 min for the preparations of Table 3; these values have been corrected for the transit delay. Although the half-times for reduction of a_K^c and I_{sc} are usually roughly comparable, they can be quite different, depending upon the duration and magnitude of the transient stimulation of short-circuit current caused by lowering the serosal potassium concentration. For example, the half-time for reduction in a_K^c was approximately 3.5 min in Run B of Preparation II, while that for I_{sc} was about 2 min. The half-time for reduction in a_K^c was similar (3.7 min) for Run A of Preparation III; however, in that tissue, the transient stimulation of I_{sc} was so prolonged that the short-circuit current was still increased above its initial value 8 min later.

Table 3 also presents the difference in electrochemical potential for K^+ across the basolateral membrane ($\Delta\tilde{\mu}^{bl}/F$) calculated with reference to the inner bathing medium. With the exception of the value of Run IB calculated for the partially recovered state, each of the values of ($\Delta\tilde{\mu}^{bl}/F$) was ≥ 0.



+ Ouabain
Restore K⁺
Voltage Difference ($\Delta E_K^c - \Delta\Psi_c$) (mV)
75
65
55
Voltage (Reference Micropipet $\Delta\Psi_c$) (mV)
0
-20
-40
-60
Voltage (K^+-Selective Microelectrode ΔE_K^c) (mV)
0
10
20
30
40
Transepithelial Current (μA)
10
5
0
-5
A
Time
1 min



- Ouabain
Remove K⁺
Restore K⁺
Voltage Difference ($\Delta E_K^c - \Delta\Psi_c$) (mV)
75
65
55
Voltage (Reference Micropipet $\Delta\Psi_c$) (mV)
0
-20
-40
-60
Voltage (K^+-Selective Microelectrode ΔE_K^c) (mV)
0
10
20
30
40
Transepithelial Current (μA)
10
5
0
-5
B
Time
1 min

Fig. 3 (*facing page*). Effect of partial inhibition of the Na,K-exchange pump on reaccumulation of potassium. (*A*): The preparation had already been subjected to four periods of transient potassium depletion prior to the time of the record. The initial baseline value of intracellular potassium activity had been 111 mM. After exposing the inner surface of the split skin to a Ringer's solution containing 0.85 mM K^+ for 17 min, a_K^c had fallen to 74 mM. At the time of the arrow, the inner medium was changed to a Ringer's solution containing concentrations of potassium and ouabain of 3.5 mM and 10^{-3} M, respectively. The change in solution produced transient depolarization of the membrane and inhibition of the short-circuit current; intracellular potassium activity transiently declined to 67 mM. Limited recovery did subsequently occur. Intracellular potassium activity transiently returned to 75 mM. However, the recovery was not sustained, and a_K^c fell back to 70 mM by the end of the recording. The fact that short-circuit current also recovered partially suggests that the Na,K-exchange pump had been incompletely inhibited, even at the high ouabain concentration used.

(*B*): The Figure presents a continuation of recordings from the same tissue of *A*. At the arrow, the inner medium was changed to a Ringer's solution containing a potassium concentration of 0.85 mM without ouabain. Intracellular potassium activity rapidly fell to 55 mM and the short-circuit current also declined. The subsequent restoration of the inner potassium concentration did produce both a slight increase in short-circuit current and a recovery in a_K^c to 74 mM. However, with the continued effects of ouabain, a_K^c did not significantly rise above the value calculated at the onset of the record in *A* for the tissue in the potassium-depleted state

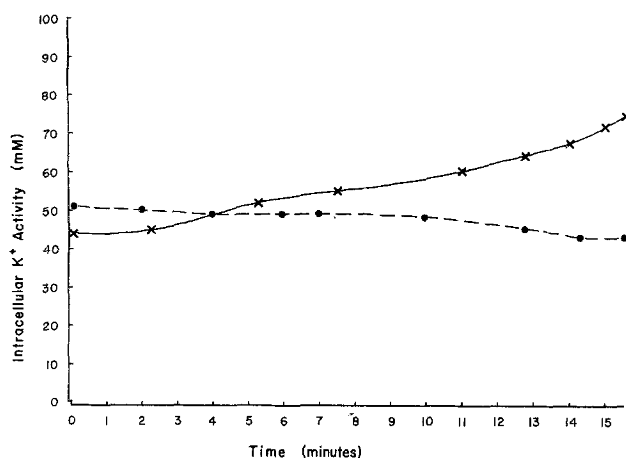


Fig. 4. Potassium reaccumulation in the presence and absence of full inhibition of the Na,K-exchange pump. The two curves present the time courses of intracellular potassium activity after the potassium concentration of the inner medium had been returned to 3.5 mM from an initial level of 0.85 mM. Both graphs are based upon data obtained from the same split skin at different times. The conditions were identical in the two cases, except that in one experiment the potassium repletion was performed in the presence of 10^{-2} M ouabain. Each curve has been generated from nine data points (including the initial and final values); the interpolated values were obtained with a parametric spline method utilizing continuous second derivatives available in a standard software package (SAS Institute, Inc., Cary, NC). Potassium was restored to the tissue after comparable degrees of potassium depletion. In the absence of ouabain, intracellular potassium activity rose from 44 to 73 mM over a period of 15 min. In the presence of ouabain, a_K^c continued to decline from 51 to 43 mM over a similar period of time

The mean ± SEM for the difference in electrochemical potential was 22 ± 4 mV, documenting that potassium accumulation proceeds against an electrochemical gradient.

The inhibition of transepithelial sodium transport by blocking the Na,K-exchange pump also inhibits reaccumulation of potassium following transient potassium depletion. This point was documented in the course of three experiments. Figure 3 presents the recorded results from an experiment in which 10^{-3} M ouabain produced incomplete inhibition of the sodium pump. The subsequent restoration of serosal potassium produced an increase in short-circuit current and an increase in a_K^c; however, the reaccumulation of potassium was insubstantial and transient. When in other experiments, the sodium pump was completely blocked with ouabain, restoration of serosal potassium produced no increase in intracellular potassium activity whatsoever. This point is particularly well illustrated by Fig. 4, which presents the reduced data calculated for two experimental periods studied with the same split skin. In each case, serosal potassium was restored to the previously potassium-depleted tissue. The degrees of potassium depletion were similar. In the absence of inhibitor, increasing c_K^s from 0.85 to 3.5 mM caused a_K^c to rise from 44 to 73 mM over the succeeding 15 min. In the presence of 10^{-2} M ouabain, the same increase in serosal potassium was associated with a continued fall in intracellular potassium from 51 to 43 mM over the same time period.

In contrast to the results observed following inhibition of the Na,K-exchange pump, inhibition of short-circuit current by blocking apical sodium entry did not appear to interfere with reaccumulation of potassium. Figure 5 presents the data recorded with one split skin. Potassium depletion had been initiated with $c_K^s = 0.85$ mM 2 hr before the beginning of the traces shown. Amiloride was added to the mucosal medium to a final concentration of 10^{-4} M 38 min after the reduction in serosal potassium concentration. Following three transient restorations of serosal potassium, c_K^s was returned to 3.5 mM at the time of the arrow. At that point, the intracellular potassium activity was cal-

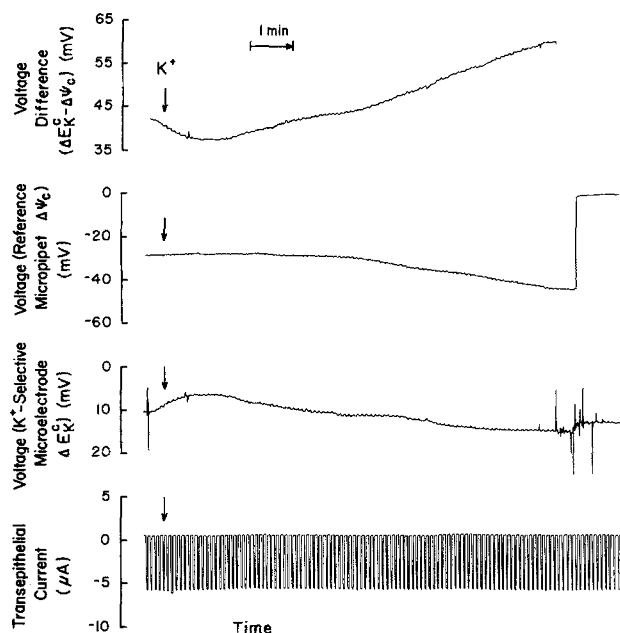


Fig. 5. Potassium reaccumulation despite inhibition of sodium entry. The potassium concentration of the inner perfusing solution had been reduced from 3.5 to 0.85 mM 2 hr before the time indicated by the arrow. After potassium depletion for 38 min, 10^{-4} M amiloride was added to the outer solution and retained in the outer medium during the remainder of the study. During the course of three initial unsuccessful attempts to place and maintain the microelectrode and micropipette within cells simultaneously, the inner potassium concentration had been alternately restored to 3.5 mM and reduced to 0.85 mM, once again. The minimum value observed for the intracellular potassium activity was 14 mM. After potassium was again restored to the inner medium at the time of the arrow, a^c_K displayed a sustained increase with time, reaching 34 mM by the end of the record. The short-circuit current was not altered by the addition of potassium, consistent with the thesis that amiloride had blocked apical sodium entry

culated to be 14 mM. Over the succeeding 8 min, a^c_K rose to a value of 34 mM, despite the continued presence of amiloride in the outer solution. A similar result was obtained with a second split skin treated in the same way. Despite the presence of 10^{-4} M amiloride in the outer bathing medium, returning the serosal potassium concentration to 3.5 mM from an initial value of 0.85 mM produced an increase in a^c_K from a depleted level of 17 to 64 mM over a period of 17 min.

Discussion

The present manuscript further documents the existence of a time-lag phenomenon noted with the urinary bladder of the toad. Following potassium depletion, the intracellular potassium activity (a^c_K) and short-circuit current are depressed. Restoration of serosal potassium produces increases in both parameters, but the rise in a^c_K preceded that in short-circuit current by 21–26 min. The initial description of the phenomenon had been based on separate measurements of ΔE^c_K and $\Delta\psi_c$ (DeLong & Civan, 1978). The data reported now indicate that the phenomenon can be observed even when measurements are performed with double-barrelled microelectrodes.

The simplest and most direct interpretation of the phenomenon is that restoration of serosal potassium permits the reaccumulation of cell potassium by the sodium pump in exchange for the extrusion of cell sodium before significant amounts of mucosal sodium can enter the cell across the apical plasma membrane. This concept is supported by the reports that: (i) Sufficient intracellular sodium is accumulated during potassium depletion of toad bladder epithelium to support such exchange (Civan et al. 1980). (ii) Negative feedback between intracellular sodium activity a^c_{Na} and apical sodium permeability (P^{ap}_{Na}) could provide the basis for the reduced apical sodium entry following potassium depletion (Erlij & Smith, 1973; Lewis, Eaton & Diamond, 1976; Cuthbert & Shum, 1977; Turnheim, Frizzell & Schultz, 1978; Weinstein et al., 1980; Chase & Al-Awqati, 1979); this feedback may be mediated by changes in intracellular calcium activity (Blaustein, 1974; Grinstein & Erlij, 1978; Taylor & Windhager, 1979; Lee, Taylor & Windhager, 1980; Chase & Al-Awqati, 1981), and (iii) Computer simulation has permitted qualitative replication of the time-lag phenomenon, once the feedback between a^c_{Na} and P^{ap}_{Na} is introduced into the model (Civan & Bookman, 1982). Since these pieces of evidence are indirect, a more direct testing of the thesis was attempted in the present work.

Clearly, it would have been preferable to have continued the experimental work with the same tissue, toad bladder. However, we and most other investigators in the field have been unable to achieve the degree of stability of intracellular recording with this tissue (illustrated by Figs. 3–5) which we have considered necessary for the present purposes. For this reason, most of the work has been continued with split frog skin, a preparation with similar transport properties, but far more satisfactory for electrophysiological study. With this tissue, it has been possible to monitor intracellular potassium activity and electrical potential in cells during the course of potassium depletion and repletion. In the present study, it has been possible to maintain the intracellular position of the microelectrode tip for as long as 2 hr within the same cell. Of central interest has been the effects induced by restoring serosal potassium to depleted tissues

while apical sodium entry has been blocked. At a mucosal concentration of 10^{-4} M, amiloride exerts its maximal effect to inhibit apical sodium entry both in frog skin and toad bladder (Erlij & Smith, 1973; Cuthbert & Shum, 1974; Sudou & Hoshi, 1977). At this concentration, amiloride did not prevent frog skin from reaccumulating potassium (Fig. 5). Amiloride may not, of course, have decreased the apical sodium permeability entirely to zero. However, apical sodium entry must have been much reduced; in the presence of amiloride, restoration of serosal potassium did not increase short-circuit current during the period that reaccumulation of potassium was stimulated.

The question may be raised whether the dissociation of potassium reaccumulation from transepithelial sodium transport may not reflect the possibility that reuptake of potassium is largely a passive process, in response to a favorable electrochemical gradient. The current results demonstrate that such an interpretation cannot be correct. Under the baseline conditions of the present work, potassium was reaccumulated against an electrochemical gradient ($\Delta\tilde{\mu}_K^{bl}/F$) of 22 ± 4 mV. Furthermore, when the Na,K-exchange pump was completely blocked with 10^{-2} M ouabain, potassium reaccumulation was entirely inhibited (Fig. 4). These data are consistent with observations reported for renal cortical tubules. Restoration of extracellular potassium stimulates both potassium uptake and oxygen consumption; both effects are inhibited by the addition of ouabain (Harris, Balaban & Mandel, 1980).

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References

Aceves, J., Erlij, D. 1971. Sodium transport across the isolated epithelium of the frog skin. *J. Physiol. (London)* **12:**195–210

Acker, H. 1978. Measurements of potassium changes in the cat carotid body under hypoxia and hypercapnia. *Pfluegers Arch.* **375:**229–232

Biber, T.U.L., Aceves, J., Mandel, L.J. 1972. Potassium uptake across serosal surface of isolated frog skin epithelium. *Am. J. Physiol.* **222:**1366–1373

Blaustein, M.P. 1974. The interrelationship between sodium and calcium fluxes across cell membranes. *Rev. Physiol. Biochem. Pharmacol.* **70:**33–82

Candia, O.A., Zadunaisky, J.A. 1972. Potassium flux and sodium transport in the isolated frog skin. *Biochim. Biophys. Acta* **255:**517–529

Chase, H.S., Jr., Al-Awqati, Q. 1979. Removal of ambient K^+ inhibits net Na^+ transport in toad bladder by reducing Na^+ permeability of the luminal border. *Nature (London)* **281:**494–495

Chase, H.S., Jr., Al-Awqati, Q. 1981. Regulation of the sodium permeability of the luminal border of toad bladder by intracellular sodium and calcium: Role of sodium-calcium exchange in the basolateral membrane. *J. Gen. Physiol.* **77:**693–712

Civan, M.M., Bookman, R.J. 1982. Transepithelial Na^+ transport and the intracellular fluids: A computer study. *J. Membrane Biol.* **65:**63–80

Civan, M.M., Hall, T.A., Gupta, B.L. 1980. Microprobe study of toad urinary bladder in absence of serosal K^+. *J. Membrane Biol.* **55:**187–202

Civan, M.M., Hoffman, R.E. 1971. Effect of aldosterone on electrical resistance of toad bladder. *Am. J. Physiol.* **220:**324–328

Curran, P.F., Cereijido, M. 1965. K fluxes in frog skin. *J. Gen. Physiol.* **48:**1011–1033

Cuthbert, A.W., Shum, W.K. 1977. Does intracellular sodium modify membrane permeability to sodium ions? *Nature (London)* **266:**468–469

Cuthbert, A.W., Shum, W.K. 1974. Amiloride and the sodium channel. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **281:** 261–269

DeLong, J., Civan, M.M. 1978. Dissociation of cellular K^+ accumulation from net Na^+ transport by toad urinary bladder. *J. Membrane Biol.* **42:**19–43

DeLong, J., Civan, M.M. 1979. Intracellular potassium activity associated with potassium depletion from toad urinary bladder. *Colloq. Inst. Nat. Santé Rech. Med.* **85:**221–229

DeLong, J., Civan, M.M. 1980. Intracellular chemical activity of potassium in toad urinary bladder. *Curr. Top. Membr. Transp.* **13:**93–105

DeLong, J., Civan, M.M. 1983. Microelectrode study of K^+ accumulation by tight epithelia: I. Baseline values of split frog skin and toad urinary bladder. *J. Membrane Biol.* **72:**183–193

Erlij, D., Smith, M.W. 1973. Sodium uptake by frog skin and its modification by inhibitors of transepithelial sodium transport. *J. Physiol. (London)* **228:**221–239

Essig, A. 1965. Active sodium transport in toad bladder despite removal of serosal potassium. *Am. J. Physiol.* **208:**401–406

Essig, A., Leaf, A. 1963. The role of potassium in active transport of sodium by the toad bladder. *J. Gen. Physiol.* **46:**505–515

Finn, A.L., Handler, J.S., Orloff, J. 1967. Active chloride transport in the isolated toad bladder. *Am. J. Physiol.* **213:**179–184

Fisher, R.S., Erlij, D., Helman, S.I. 1980. Intracellular voltage of isolated epithelia of frog skin: Apical and basolateral cell punctures. *J. Gen. Physiol.* **76:**447–453

Giebisch, G., Sullivan, L.P., Whittembury, G. 1973. Relationship between tubular net sodium transport and peritubular potassium uptake in the perfused *Necturus* kidney. *J. Physiol. (London)* **230:**51–74

Grinstein, S., Erlij, D. 1978. Intracellular calcium and the regulation of sodium transport in the frog skin. *Proc. R. Soc. London B* **202:**353–360

Harris, S.I., Balaban, R.S., Mandel, L.J. 1980. Oxygen consumption and cellular ion transport: Evidence for adenosine triphosphate to O_2 ratio near 6 in intact cell. *Science* **208:**1148–1150

Higgins, J.T., Jr., Gebler, B., Frömter, E. 1977. Electrical properties of amphibian urinary bladder epithelia. II. The cell potential profile in *Necturus maculosus*. *Pfluegers Arch.* **371:**87–97

Kimura, G., Fujimoto, M. 1977. Estimation of the physical

state of potassium in frog bladder cells by ion exchanger microelectrode. *Jpn. J. Physiol.* **27**:291–303

Kimura, G., Urakabe, S., Yuasa, S., Miki, S., Takamitsu, Y., Orita, Y., Abe, H. 1977. Potassium activity and plasma membrane potentials in epithelial cells of toad bladder. *Am. J. Physiol.* **232**:*F*196–*F*200

Leaf, A. 1965. Transepithelial transport and its hormonal control in toad bladder. *Ergeb. Physiol. Biol. Chem. Exp. Pharmacol.* **56**:215–263

Lee, C.O., Taylor, A., Windhager, E.E. 1980. Cytosolic calcium activity in epithelial cells of *Necturus* kidney. *Nature (London)* **287**:859–861

Lewis, S.A., Eaton, D.C., Diamond, J.M. 1976. The mechanism of Na^+ transport by rabbit urinary bladder. *J. Membrane Biol.* **28**:41–70

Lindemann, B. 1975. Impalement artifacts in microelectrode recordings of epithelial membrane potentials. *Biophys. J.* **15**:1161–1164

Nagel, W. 1976. Intercellular junctions of frog skin epithelial cells. *Nature (London)* **264**:469–471

Nagel, W., Garcia-Diaz, J.F., Armstrong, W.McD. 1981. Intracellular ionic activities in frog skin. *J. Membrane Biol.* **61**:127–134

Nellans, H.N., Schultz, S.G. 1976. Relations among transepithelial sodium transport, potassium exchange and cell volume in rabbit ileum. *J. Gen. Physiol.* **68**:441–463

Nelson, D.J., Ehrenfeld, J., Lindemann, B. 1978. Volume changes and potential artifacts of epithelial cells of frog skin following impalement with microelectrodes filled with 3 M KCl. *J. Membrane Biol.* **Special Issue**:91–119

Rick, R., Dörge, A., Arnim, E. von, Thurau, K. 1978. Electron microprobe analysis of frog skin epithelium: Evidence for a syncytial sodium transport compartment. *J. Membrane Biol.* **39**:313–331

Robinson, B.A., Macknight, A.D.C. 1976*a*. Relationships between serosal medium potassium concentration and sodium transport in toad urinary bladder: I. Effects of different medium potassium concentrations on electrical parameters. *J. Membrane Biol.* **26**:217–238

Robinson, B.A., Macknight, A.D.C. 1976*b*. Relationships between serosal medium potassium concentration and sodium transport in toad urinary bladder: II. Effects of different medium potassium concentration on epithelial cell composition. *J. Membrane Biol.* **26**:239–268

Robinson, B.A., Macknight, A.D.C. 1976*c*. Relationships between serosal medium potassium concentration and sodium transport in toad urinary bladder. III. Exchangeability of epithelial cellular potassium. *J. Membrane Biol.* **26**:269–286

Sudou, K., Hoshi, T. 1977. Mode of action of amiloride in toad urinary bladder: An electrophysiological study of the drug action on sodium permeability of the mucosal border. *J. Membrane Biol.* **32**:115–132

Taylor, A., Windhager, E.E. 1979. Possible role of cytosolic calcium and Na – Ca exchange in regulation of transepithelial sodium transport. *Am. J. Physiol.* **236**:*F*505–*F*512

Turnheim, K., Frizzell, R.A., Schultz, S.G. 1978. Interaction between cell sodium and the amiloride-sensitive sodium entry step in rabbit colon. *J. Membrane Biol.* **39**:233–256

Ussing, H.H. 1960. The alkali metal cations in isolated systems and tissues. *In:* The Alkali Metal Cations in Biology. H. Ussing, P. Kruhoffer, J.H. Thaysen, and N.A. Thorn, editors. Vol. 13, pp. 1–195. Springer-Verlag, Berlin – Heidelberg – Göttingen

Weinstein, F.C., Rosowski, J.J., Peterson, K., Delalic, Z., Civan, M.M. 1980. Relationship of transient electrical properties to active sodium transport by toad urinary bladder. *J. Membrane Biol.* **52**:25–35

Valenzeno, D.P., Hoshiko, T. 1977. Potassium reaccumulation by isolated frog epidermis. *Biochim. Biophys. Acta* **470**:273–289

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