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Voltage dependent membrane conductances in cultured renal distal cells

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Cultured Na^+-transporting epithelia from amphibian renal distal tubule (A6) were impaled with microelectrodes and analyzed at short-circuit and after transepithelial voltage perturbation to evaluate the influence of voltage on apical and basolateral membrane conductances. For equivalent circuit analysis, amiloride was applied at each setting of transepithelial potential. At short-circuit, apical and basolateral membrane conductances averaged 88 and 497 $\mu S/cm^2$, respectively ($n = 10$). Apical membrane conductance, essentially due to Na^+-specific pathways, decreased after depolarization of the apical membrane. The drop was considerably larger than predicted by the Goldman-Hodgkin-Katz (GHK) constant-field equation. This suggests decrease in permeability of the apical Na^+ channels upon depolarization. Basolateral membrane conductance, preferentially determined by K^+ channels, increased after hyperpolarization of the basolateral membrane. This behavior is contrary to the prediction of the GHK constant field equation and reflects inward rectification of the K^+ channels. The observed rectification patterns can be valuable for maintenance of cellular homeostasis.

Introduction

A6 cells, cultured from the renal distal tubule of *Xenopus laevis* [1], can develop as a tight homogeneous monolayer, which transports Na^+ actively from apical to basolateral side. As in most other tight Na^+-transporting epithelia, transcellular conductance is predominantly determined by the apical membrane Na^+ conductance, g_a [2]. Depolarization of the epithelial cells by exposure of the basolateral side to high K^+ concentrations or Ba^{2+} was associated by a decrease in g_a. This decrease was larger than the response predicted for the observed change in membrane potential from the Goldman-Hodgkin-Katz (GHK) constant-field equation for rectification in Na^+-specific channels. Tentatively, we explained these effects by voltage sensitivity of the apical Na^+-channels. Since the duration of experimental perturbations was rather long in the above study, other factors such as redistribution of intracellular ions or influences from intracellular messengers could not be excluded. Furthermore, due to the experimental protocol, possible voltage responses of basolateral K^+ channels, which account for more than 70% of conductance in this membrane, could not be discovered at all. To obtain more direct information on the influence of voltage on both membrane conductances in A6 cells, the response of specific membrane conductances on brief perturbation of the transepithelial potential was analyzed. The present data corroborate our view that apical membrane Na^+-permeability decreases with depolarization. In addition, we noticed voltage sensitivity of the basolateral membrane conductance, with similar characteristics as reported for inward rectifying K^+-specific channels of other tight epithelia [3–5]. These patterns of apical and basolateral membrane conductances, which make up negative feed-back systems, might be valuable for the control of transepithelial Na^+ transport.

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Methods

A6 cells were selected from American Type Culture Collection (Bethesda, U.S.A.). Growth conditions were previously described in detail [2]. The cells were replated with a density of approx. $2 \cdot 10^6$ cells/cm^2 on

permeable nitrocellulose ester membranes (Millipore PIHA 030050) and were fed three times a week. After 15–18 days, monolayers were carefully punched out and mounted in a modified Ussing-type chamber (0.4 cm^2 tissue area) for microelectrode experiments [6]. The edge area of the apical half chamber was covered with heavy silicone grease to avoid edge damage. Apical and basolateral side were continuously and separately superfused with a solution of the following composition (in mM): NaCl, 115; $KHCO_3$, 3.5; $CaCl_2$, 1; NaH_2PO_4, 0.9; $MgCl_2$, 1; pH was adjusted to 7.5. Flow rates, achieved by hydrostatic pressure differences, were about 10 and 2–3 ml/min on apical and basolateral side, respectively. Perfusion solutions could be changed on either side by means of non-interrupting valves. Transepithelial potential difference (V_t) was measured with reference to the apical side using calomel electrodes connected to the tissue chamber via KCl-agar bridges. Circular silver wires 5 mm apart from the tissue surface served for sending current from an automatic voltage clamp. Under baseline conditions, the tissue was short-circuited and the short-circuit current (I_{sc}) was recorded. Brief perturbations ($\Delta V_t = 20$ mV, 400 ms every 2 s) were used to determine tissue conductance (G_t) from the change in transepithelial current (I_t) ($G_t = \Delta I_t / \Delta V_t$) and fractional apical resistance (fR_a) from the change in apical membrane voltage (V_a) ($fR_a = R_a / R_c = \Delta V_a / \Delta V_t$). R_a and R_c refer to apical membrane and transcellular resistance, respectively. Microelectrodes were prepared from filamented borosilicate glass capillaries. Filled with 1 M KCl, input resistance was > 50 MΩ. Monolayers were impaled from the apical side with the aid of a stepping motor micromanipulator. V_a was measured with reference to the apical side. For convenience, basolateral membrane potential, V_b, is reported with reference to the basolateral side; the intracellular potential under short-circuit conditions is denoted by V_{sc}. Electrical parameters were monitored on a multichannel strip chart recorder. In addition, the data were stored on a PC-compatible computer at a sample rate of 2 s using a 14 bit analog/digital converter. Impalements, evaluated according to the criteria described previously [2], yielded stable readings for up to 1 h.

Apical and basolateral membrane conductances (g_a and g_b) were derived using the equivalent circuit equations [7]:

$$g_a = (G_t - G_t')/fR_a \quad (1)$$

$$g_b = (G_t - G_t')/(fR_a' - fR_a) \quad (2)$$

Symbols with ' indicate values in the presence of 10^{-5} M amiloride on the apical side. Since transepithelial voltage perturbations were associated by unpredictable changes in shunt conductance (see below), the effect of perturbing V_t on apical and basolateral parameters was analyzed as described previously [7]. In brief, V_t was perturbed from the short-circuited state to values of ± 34 and ± 68 mV. After an equilibration period of 20–25 s, 10^{-5} M amiloride was applied to determine G_t' as a measure of the shunt conductance. The tissue was then again short-circuited and thereafter amiloride was washed out. After stabilization of the electrical parameters at values close to the preceding control period, V_t was clamped to a new level and the procedure was repeated. Data immediately before and 20 s after addition of amiloride were used for equivalent circuit analysis. Since the values of g_a and g_b are derived from perturbations of small magnitude and brief duration, they reflect the slopes at the respective membrane potentials. Theoretical responses of g_a and g_b at constant membrane permeabilities were calculated from the Goldman-Hodgkin-Katz (GHK) constant field equation for each transepithelial perturbation. Slopes at the observed membrane potentials were computed and related to the respective values at short-circuit. Ion concentrations in the perfusion solution are considered for the extracellular space; intracellular concentrations as in typical amphibian epithelia and identical activity coefficients in intra- and extracellular spaces were assumed.

Response of basolateral membrane conductance on variation of the I_{sc} was also qualitatively assessed using the relationship:

$$V_{sc} = E_b - R_b \cdot I_{sc} \quad (3)$$

where E_b and R_b reflect zero current potential and resistance of the basolateral membrane, respectively. Values were read 15–20 s after addition of different concentrations of amiloride, which led to progressive inhibition of the I_{sc}. A time lag of 15–20 s was found to be necessary for the approach of steady values of I_{sc}. The resulting correlation between I_{sc} and V_{sc} represents an I/V relationship of the basolateral membrane. Since E_b and R_b are likely affected by the protocol, numerical analysis of the data would not be meaningful. Nevertheless, qualitative information on transport-related alteration of these both parameters can be deduced.

Amiloride HCl was a generous gift of Dr. G. Fanelli (Merck, Sharp and Dohme; West Point, PA). Mean values are presented $\pm$S.D. Significance of differences is calculated using Student's t-test.

Results

After transfer of the monolayers to the chamber and incubation at short-circuit for about 15 min, I_{sc} and G_t stabilized at values averaging 6.7 ± 2.1 $\mu A/cm^2$ and

321 ± 81 $\mu S/cm^2$, respectively ($n = 10$). Application of 10^{-5} M amiloride decreased I_{sc} and G_t to 0.3 ± 0.4 $\mu A/cm^2$ and 249 ± 79 $\mu S/cm^2$, respectively. V_{sc} averaged -51 ± 12 mV in the Na^+-transporting state and increased after amiloride to -64 ± 16 mV, whereas fR_a increased from 0.82 ± 0.07 under Na^+-transporting conditions to 0.98 ± 0.02 after amiloride. Specific membrane conductances g_a and g_b as calculated from Eqns. 1 and 2 were, on the average, 88 ± 27 and 497 ± 208 $\mu S/cm^2$, respectively.

The influence of perturbing V_t to four different values between +68 mV and −68 mV on apical and basolateral membranes is shown for a typical experiment in Fig. 1. At each level of V_t, amiloride was briefly applied approx. 20 s after onset of the voltage perturbation. It is evident that fR_a increased almost instantaneously after perturbation to positive values of V_t, which depolarized the apical membrane. Perturbation to negative values of V_t had the opposite effect. The changes in fR_a were more pronounced at larger magnitudes of voltage perturbation. After the addition of amiloride, fR_a increased in all cases rapidly to values near 1.0. G_t declined after perturbation to positive values of V_t. Perturbation to the negative direction led to increase in G_t. The effects were larger after perturbation to the higher values of V_t. It can be derived from the responses of G_t on application of amiloride that the decrease in conductance was restricted to the transcellular pathway for both positive values of V_t; amiloride-insensitive shunt conductance remained essentially unchanged in the depicted experiment. During perturbation to negative values of V_t, the shunt conductance increased slightly. This is particularly evident for the return from perturbed V_t to short-circuit. In other experiments, larger alteration of the shunt conductance with negative and positive going

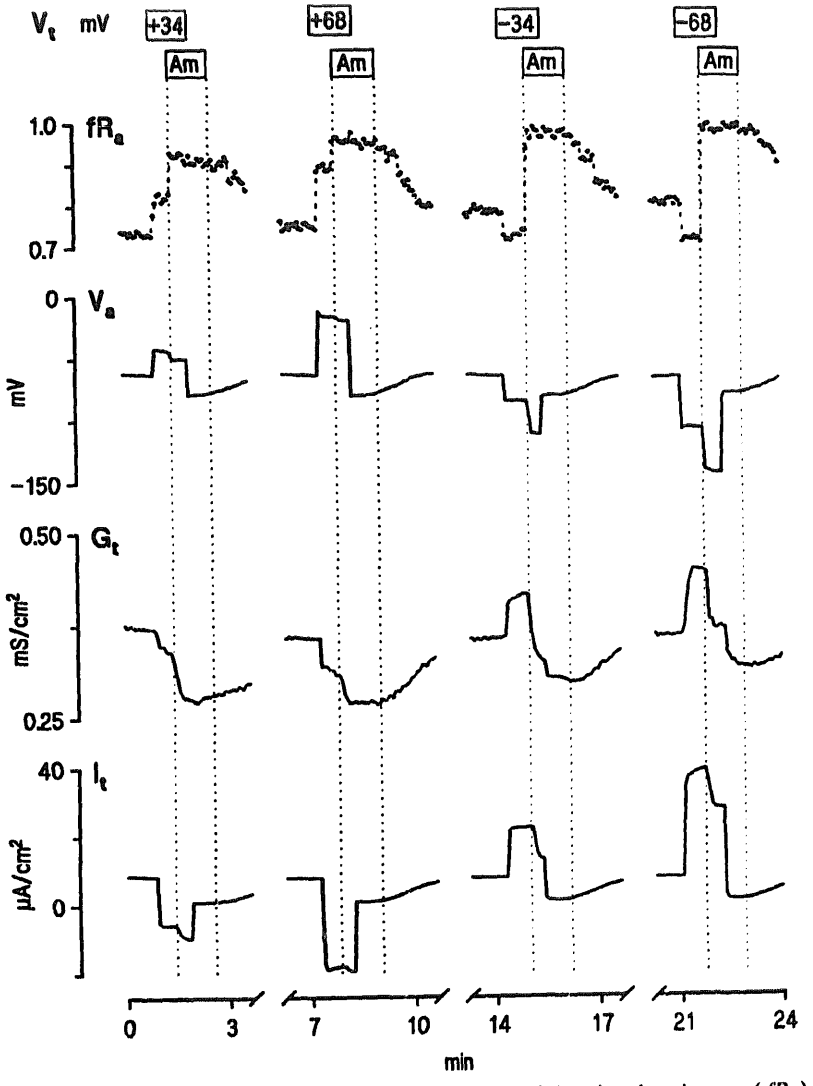

Fig. 1. Response pattern of transepithelial current (I_t) and conductance (G_t), apical fractional resistance (fR_a) and membrane potential (V_a) during perturbation of transepithelial potential difference (V_t) in a A6 cell monolayer. The level of V_t d apical perfusion with 10^{-5} M amiloride (Am) are indicated in the bars at the top. The dashed line point to addition to and removal of amiloride from the apical perfusion solution. Note the near-instantaneous response of fR_a after voltage perturbation.

voltages was noted. Nevertheless, the major fraction of change in G_t during voltage perturbation was in all cases localized to the transcellular limb. At $V_t = +68$ mV, amiloride had minimal effects on I_t and V_a. Since fR_a increased to about the same level as during short-circuit and G_t declined, the apical membrane Na^+-channels were unquestionably affected by the inhibitor. With negative perturbation of V_t, amiloride induced larger changes of I_t; this was associated by increased hyperpolarization of V_a.

Results of ten experiments are summarized in Table I. Values of g_a and g_b during voltage perturbation are normalized to the respective values at short-circuit immediately before the perturbation to account for spontaneous alterations with time and to reduce variations between individual experiments. It appears that amiloride-sensitive current (dI_{am}) as well as normalized apical membrane conductance (g_a^*) increased considerably in direction from positive to negative values of V_t, whereas the normalized basolateral membrane conductance (g_b^*) decreased concomitantly. Comparison of the measured values with GHK-predicted fractional changes in conductances (Table I) reveals that conductances of both membranes deviate from the behavior predicted for constant permeability.

In the case of g_a (apical membrane Na^+-specific channels), the observed decrease in conductance coincided with the direction of the GHK-predicted change for constant membrane permeability; the magnitude of decrease, however, was considerably larger than calculated. Accordingly, the data suggest decrease in Na^+ permeability with depolarization of the membrane. This impression is corroborated by Fig. 2, which shows the correlation between g_a and V_a of the individual experiments. In all experiments, an almost linear decrease in g_a was obtained in the range of depolarized apical membrane. Hyperpolarization of the apical membrane led only in some experiments to further increase in g_a; in most cases, g_a was at a saturating level under short-circuit conditions.

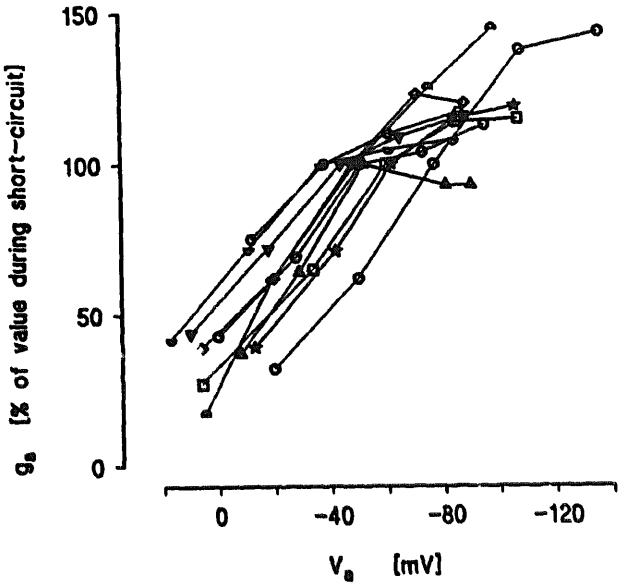

Fig. 2. Normalized apical membrane conductance (g_a^*) as a function of apical membrane potential (V_a) during perturbation of V_t from the short-circuited state to ±34 and ±68 mV. Data are normalized to g_a at short-circuit. Results of individual experiments are indicated by different symbols and connected by lines.

The increase in g_b with hyperpolarization of V_b appears less pronounced, but it must be considered that the values of V_b span a comparatively small range. Nevertheless, the data from the individual experi-

TABLE I

Effect of perturbations of transepithelial voltage (V_t) on amiloride-sensitive current (dI_{am}), apical and basolateral membrane potential (V_a, V_b), and on normalized conductances (g_a^*, g_b^*)

Membrane conductances relative to the values at short-circuit (g_a^{GHK}, g_b^{GHK}) were calculated for constant ion permeability from the GHK constant-field equation. Intracellular concentrations of 8 and 120 mM for Na^+ and K^+, respectively, and identical activity coefficients in extra- and intracellular spaces are assumed.

V_t (mV)	dI_{am} ($\mu A/cm^2$)	V_a (mV)	g_a^* (%)	g_a^{GHK} (%)	V_b (mV)	g_b^* (%)	g_b^{GHK} (%)
+68	1.3	−2	39	67	−68	128	71
$n = 6$	±0.3	±10	±6		±11	±22	
+34	3.3	−26	67	85	−60	116	83
$n = 10$	±1.4	±12	±5		±12	±12	
0	6.4	−51	100	100	−51	100	100
$n = 10$	±2.0	±12			±12		
−34	8.9	−76	114	110	−42	79	119
$n = 10$	±2.9	±13	±12		±13	±7	
−68	11.1	−99	122	116	−31	70	145
$n = 9$	±4.2	±15	±12		±15	±6	

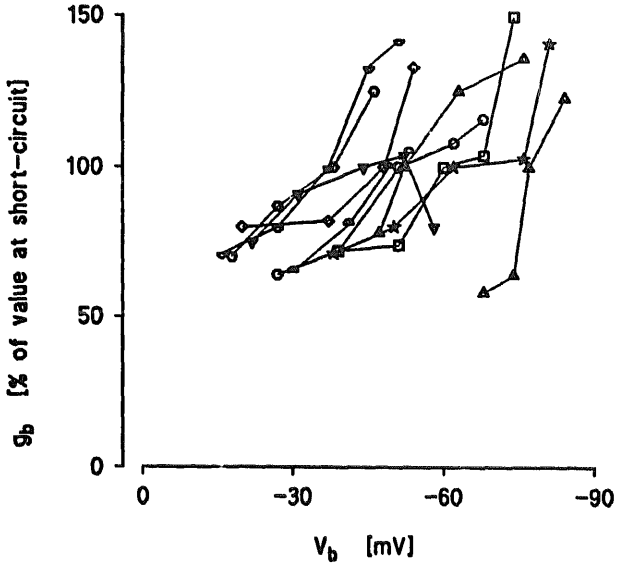

Fig. 3. Normalized basolateral membrane conductance, g_b^* as a function of basolateral membrane potential, V_b, during perturbation of V_t to ±34 and ±68 mV. Data are given relative to b_b at short-circuit. Different symbols connected by lines indicate results from individual experiments.

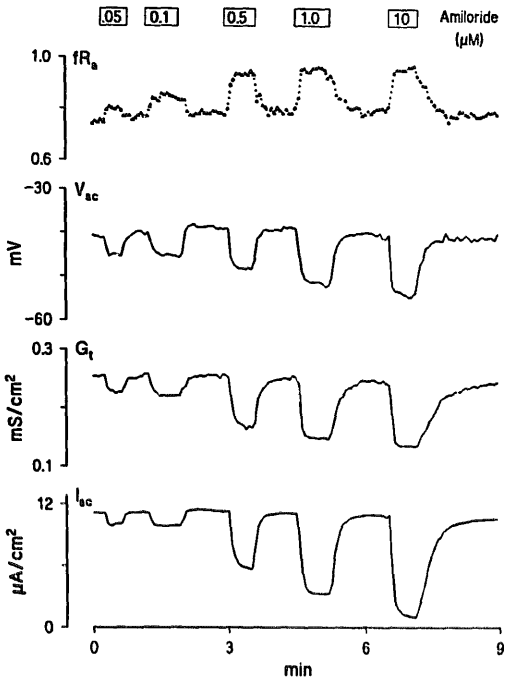

Fig. 4. Response of transepithelial and intracellular electrical parameters on application of increasing amiloride concentrations at the apical side. Amiloride concentration is indicated in the top bars.

ments, which are depicted in Fig. 3, reveal that g_b increased consistently with hyperpolarization over the whole range of voltage perturbations. This behavior is opposite in direction to the pattern, which is predicted from the GHK constant field equation for K^+-selective channels with constant permeability. Accordingly, the deviation from the predicted response of basolateral K^+-channels is even larger than for apical Na^+-channels and we have to postulate increase in basolateral permeability at hyperpolarized membrane potentials.

In view of the notable changes in g_b induced by transepithelial voltage perturbation, it was interesting to analyze whether similar influence on g_b could be elicited by the alteration of V_{sc}, which result from changes of transcellular Na^+ transport rate at short-circuit. Experimentally, stepwise inhibition of Na^+ transport was accomplished by application of gradually increasing concentrations of amiloride in the apical perfusion solution. Fig. 4 shows the electrical parameters for apical amiloride concentrations from 0.05 to 10 μM observed for a typical experiment. Progressive increase in fR_a from the control value of 0.70 was associated with gradually increasing inhibition of the apical membrane. V_{sc} hyperpolarized rapidly, whereas I_{sc} and G_t decreased to new steady values within 10 s at each amiloride concentration. After return to amiloride-free apical perfusion, all electrical parameters returned to the preceding control levels. From corresponding values of I_{sc} and V_{sc} at gradually increasing inhibition of Na^+-transport, a steady state I/V plot of the basolateral membrane can be generated. Fig. 5 shows these I/V relationships of the basolateral membrane for six monolayers with baseline Na^+ transport rate between 4 and 11.5 μA/cm². I_{sc} and V_{sc} at the spontaneous rate of transport are reflected by the dot at the lower right end of the plot, whereas the dot at the left end represents I_{sc} and V_{sc} at the highest concentration of amiloride, i.e., at Na^+-transport close to zero. The relationships were almost linear in four monolayers; the remaining two tissues

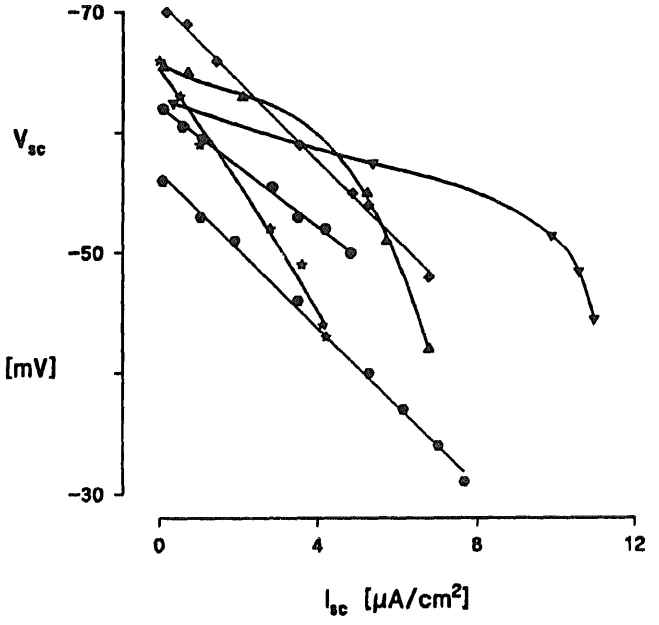

Fig. 5. I/V relationship of the basolateral membrane of A6 cell preparations obtained by sequential brief exposure of the apical side to amiloride at increasing concentrations between $3 \cdot 10^{-8}$ and 10^{-5} M. Lines connect data from individual tissues.

exhibited non-linearity with decreasing slope at depressed I_{sc}. Since the slope of the relationships reflect the reciprocal of basolateral conductance (Eqn. 3), it can be derived that g_b remained constant or increased when the I_{sc} was reduced. This pattern is opposite to the prediction from the GHK constant field equation for basolateral K^+-channels with constant permeability. It agrees, on the other hand, with our results from transepithelial voltage perturbation and indicates that the response reflects transport-related reactions of the basolateral membrane.

Discussion

In a recent study we observed that the Na^+-specific apical membrane conductance of A6 cells decreased when the apical membrane was depolarized by basolateral perfusion with high K^+ concentrations or Ba^{2+} [2]. The depression was considerably larger than the response upon the change in membrane potential predicted from GHK constant field rectification. Our data demonstrate that depolarization of the apical membrane by transepithelial voltage perturbation leads to a similar decrease in g_a. The time-course of the changes in g_t and fR_a after perturbation of V_t indicates that the response of g_a occurred within the first 2–4 s. Notable alteration of intracellular Na^+ concentration is very unlikely at this rather early time. Furthermore, it can be shown that the deviation between measured and GHK predicted values of g_a is only slightly reduced, if maximally possible changes in intracellular Na^+ concentration due to continuing pump activity after voltage perturbation and before application of amiloride are considered. It should be noted that the lag between addition of amiloride and reading of electrical parameters does not influence data of the cellular limb. Values after amiloride, required to estimate the shunt conductance, cannot be obtained before homogeneous distribution of the blocker in the chamber, which is limited by the rate of perfusion. The perfusion rate, however, could not be increased without endangering the stability of microelectrode impalements. Due to time and voltage dependent alteration, shunt conductance, G_t', could not be determined once at the end of the experimental period, but had to be estimated at each level of V_t. Although this protocol is considerably more laborious and time consuming than the usually applied technique, we consider electrical parameters derived in this way much more meaningful and reliable.

From the data, we have to conclude that the apical Na^+-permeability decreased with depolarization of the membrane. This observation corroborates our previous view [2] that the response of g_a to basolateral application of high K^+ concentrations or Ba^{2+} did not represent indirect effects, but rather reflects inherent properties of the apical Na^+ channels. The voltage sensitivity of apical Na^+-channels could be physiologically advantageous, since it represents a negative feed-back. High rate of Na^+ entry is usually associated with depolarization of the cells. If reduction of the Na^+ permeability ensues from this depolarization, it will limit the increase in Na^+ influx in addition to the decrease in driving force. This may protect the (Na^+/K^+)-ATPase against excessively high transport rates. Further, Na^+ entry across the apical membrane can be more effectively adjusted with respect to the conditions for recirculation of K^+ across the basolateral border, because the latter affects V_a and consequently driving force across the membrane and permeability for Na^+.

Our conclusion is at variance with two previous analyses demonstrating (i) voltage independence of conductance and gating of apical Na^+ channels from A6 cells reincorporated in planar lipid bilayer [8] and (ii) a near linear I/V relationship of open channels over a range of ± 80 mV and almost no voltage-dependency of mean open/closed time in single channel recordings [9]. It appears possible that some features of the in situ channel were lost during isolation or that the applied techniques for isolating channels extracted a normally quiescent type. Similar views were recently formulated by Wills and Millinoff [10], who observed that the cellular pathway is highly selective for Na^+ over K^+, in contrast to poor $Na^+:K^+$ selectivity reported in studies of the amiloride-sensitive channels from A6 apical membranes using either the patch-clamp technique [9] or the incorporation of apical membrane vesicles into planar lipid bilayer [8], and was also expressed by Palmer et al. [11], who analyzed characteristics of Na^+ channels incorporated in *Xenopus* oocytes by injection of mRNA from A6 cells. The voltage sensitivity of these highly selective Na^+ channels agrees with that of the present study.

The negative intracellular potential recorded in A6 cells arises mainly from K^+ diffusion across the basolateral barrier, whose conductance is predominantly accounted for by K^+-specific channels [2]. The basolateral membrane potential after blockage of Na^+ entry is considerably smaller than the likely Nernst potential for K^+. This indicates that other, yet unidentified ions must be permeable. In view of recent observations, it was particularly interesting to analyze whether the basolateral membrane of A6 cells displays similar inward rectification as reported for other tight Na^+-transporting epithelia [3–5]. Due to the small rate of Na^+ transport in non-stimulated tissues, amiloride-induced changes in V_{sc} are small and usually unsuitable to test for rectification patterns. The range of membrane potentials in A6 cells could be extended by the present protocol using transepithelial voltage perturbation, although we could not overcome the limitation

that changes in V_b are comparatively small with $fR_a > 70\%$ as in the present study. Our data indicate that g_b was directly related to the voltage-induced changes in V_b. This increase in g_b on hyperpolarization of V_b is opposite in sense to the prediction of the GHK constant field equation. The slopes of t'.e steady state I/V curves of the basolateral membrane support this conclusion: we never observed that these I/V curves were concave; relationships were either linear or convex and this conforms with increase in g_b after hyperpolarization of the basolateral membrane. The data in Table I show that the voltage-sensitivity of the rectifier is noticeable. Alteration of intra- or extracellular K^+ concentration, which could affect the observed deviation from the GHK prediction, is unlikely. The brief delay between perturbation and measurement suggests against disturbance of other intracellular constituents as pH, Ca^{2+}, metabolites, etc. and points to membrane potential as the principal source for the observed reactions. It cannot be revealed, whether the behavior is due to properties of the open channels or whether it results from influence on the open/close probability of the channels. One physiological implication of these rectification patterns is that the changes of the intracellular potential caused by alteration of rheogenic ion fluxes are thereby accentuated. These responses facilitate the operation of negative feed-back system, which are clearly advantageous for the regulation of transmembranal ion fluxes, since they operate against the occurrence of disbalance. A positive feed-back system, i.e., parallel increases in transport rate and basolateral K^+ conductance, associated with augmented electrochemical gradient for efflux, on the other hand, is much more susceptible to uncontrolled K^+ loss. It appears from the present and from previous results [3,5] that increase at higher rates of transepithelial transport as demonstrated in several epithelial preparations and proposed as a meaningful mechanism for membrane cross-talk (for a recent review see Ref. 12), does not necessarily and generally have to occur in all tissues. In tight epithelia, which transport predominantly Na^+ in electrogenic mode, response of the K^+ permeability may actually be opposite and valuable for regulative purposes in this sense.

Acknowledgements

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