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Relationship of Transepithelial Electrical Potential to Membrane Potentials and Conductance Ratios in Frog Skin

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Summary. Previous studies in anuran epithelia have shown that, after clamping the transepithelial voltage in symmetrical sequences for 4–6 min there is near-constancy of the rate of active Na transport and the associated oxidative metabolism, with a near-linear potential dependence of both. Here we have investigated in frog skin the cellular electrophysiological events associated with voltage clamping (V_t = inside-outside potential). Increase and decrease of V_t produced converse effects, related directly to the magnitude of V_t.

Hyperpolarization resulted in prompt decrease in inward transepithelial current I_t and increase in fractional outer membrane resistance fR_o (as evaluated from small transient voltage perturbations) and in outer membrane potential V_o. Overshoot of V_o was followed by relaxation to a quasi-steady state in minutes. Changes in fR_o were progressive, with half times of some 1–5 sec. Changes in transepithelial slope conductance g_t were more variable, usually preventing precise evaluation of the outer and inner cell membrane conductances g_o and g_i. Nevertheless, it was shown that g_o is related inversely to V_t and V_o. Presuming insensitivity of g_i to V_t, the dependence of g_o on V_o in the steady state much exceeds that predicted by the constant field equation. Apparent inconsistencies with earlier results of others may be attributable to differences in protocol and the complex dependence of g_o on V_o and/or cellular current. In contrast to previous findings in tight epithelia at open circuit, differences in V_t were associated with substantial differences in fR_o and inner membrane potential V_i. Hyperpolarization of V_t over ranges commonly employed in studies of active transport and metabolism appears to increase significantly the electrochemical work per Na ion transported.

Key words membrane potentials · membrane conductances · frog skin · constant field equation · sodium conductances

Introduction

Determinations of current-voltage relationships are commonly employed in the characterization of transepithelial active sodium transport. The nature of these relationships is importantly influenced by the magnitude, duration, and sequence of potential perturbations employed. In studies in both frog skin and toad bladder it has been found that with symmetric perturbations of transepithelial potential the rates of both transcellular active sodium transport and the associated oxidative metabolism become near-constant within about 1–4 min, suggesting the existence of quasi-steady states [38, 43]. Since at these times the dependence of both rates on potential was approximately linear [43], it was possible to characterize the tissue by a set of unique-valued parameters in terms of either an equivalent electrical circuit or linear nonequilibrium thermodynamic formulation [8]. The evaluation of these parameters permits the systematic investigation of kinetic and energetic factors influencing active transport.

The apparent simplicity of the relationships defined by the above means seems somewhat surprising when it is considered that our "black box" incorporates two series elements with very different characteristics, an outer element mediating passive entry of sodium into the cytoplasm and an inner element carrying out active transport of sodium out of the cytoplasm into the interstitial fluid. In order to characterize the behavior of these elements with voltage clamping under quasi-steady state conditions, we have here studied cellular electrical events associated with protocols similar to those employed previously for studies of active sodium transport and suprabasal oxidative metabolism.

Materials and Methods

Experiments were carried out using isolated skins of *Rana temporaria* and *R. esculenta* incubated in regular Ringer's solution (composition in mM: 110, Na; 2.5, K; 1, Ca; 112, Cl; 2.5, HCO_3; pH after equilibration with air, 8.1). Techniques for tissue preparation and microelectrode impalement were as described previously [32, 33]. The skins were continuously voltage clamped to desired levels of V_t by means of an automatic clamping device (M. Frankenberger and W. Nagel, *in preparation*). All voltage values are expressed with reference to the outer solution, which was grounded via a Ag/AgCl-electrode, and current is considered positive when flowing from outside to

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inside. Transepithelial slope conductance ($g_t = -\delta I_t/\delta V_t$) and the voltage divider ratio ($\delta V_o/\delta V_t$) were estimated every 0.5 to 10 sec from the changes in transepithelial current I_t and microelectrode potential V_o, respectively, on increment of the clamping voltage V_t by +20 mV for periods of between 100 and 250 msec. (Presuming insignificant interstitial resistance to current flow, the voltage divider ratio is here considered to approximate the fractional outer membrane resistance $fR_o = R_o/(R_o + R_i) = R_o/R_a$, where R_o and R_i are the outer and inner cell membrane resistances and R_a the transcellular resistance, respectively [4, 13].) Frequency and duration of the pulses were selected according to the particular protocol. The values of I_t and V_o were each obtained by means of two sample/hold amplifiers (Intersil IH 5110) appropriately triggered so as to sample values of I_t and V_o for 20 msec immediately before the onset and before the very end of the pulse. The differential output of the sample/hold amplifiers was calibrated with respect to the imposed V_t, thus allowing direct quasicontinuous recording of g_t and fR_o.

The values of V_t, I_t, g_t, V_o and fR_o were continuously recorded on a multichannel strip chart recorder (Servogor 960, Metrawatt, Nürnberg, BRD), which has a response time of 400 msec for full scale. Accordingly, precise display of the response to short pulses of V_t was not possible. On the other hand, the low temporal resolution of the recording system should exclude response within the time of capacitive changes of the frog skin membranes, which have been reported to range below 100 msec [5, 41]. Observation on a storage oscilloscope insured that this was indeed the case.

Criteria for accepting a particular impalement as reliable were as described previously [32, 33, 35]. In brief, in addition to requiring appropriate and near constant values of tip potential and input resistance of the microelectrode before and after impalement, impalements were accepted only if they satisfied the following criteria: (i) Near identical readings of V_o and fR_o were obtained in repeated impalements. (ii) On addition of amiloride V_o became more negative and fR_o rose to near 1.0. In a given tissue those values of V_o and fR_o are most reliable that show the biggest absolute changes on equivalent alterations of the short circuit current following the addition of amiloride. It is important to note that fR_o usually increased to above 0.98 on application of amiloride (10^{-5} M), even in those tissues that initially had extremely low values of fR_o in the short circuited state. In some experiments, on the other hand, it was observed that in obviously "good" impalements the amiloride-induced level of fR_o did not exceed 0.92. This could result from conductance of the apical plasma membrane to ions other than sodium, e.g., potassium or chloride [2]. To what extent lateral intercellular space resistance affects the accuracy of our estimates of fR_o is presently unknown. The available evidence suggests that for tight epithelia the error is unlikely to be important, even in the absence of amiloride [4]. (iii) Other factors being equal, those values of V_o were most reliable that were associated with lowest values of microelectrode input resistance during impalement [1]. Values of fR_o and changes of V_o with experimental manipulations are obviously unaffected by this factor. (iv) The recording of cellular potentials was stable within 1–2 mV, and fR_o was stable within 2–3% over periods of more than 10 min. This criterion was required only because of the long periods of study necessitated by the protocols employed here.

In some experiments we observed only I_t and g_t, in order to yield information on the potential dependence of the transcellular slope conductance g_a. The quantity g_a was estimated as the amiloride-inhibitable component of g_t at various settings of V_t. In three studies such analyses were combined with microelectrode measurements.

Results

General Nature of Response to Voltage Perturbation

Our basic purpose was to determine the dependence on the transepithelial electrical potential difference V_t of the transepithelial current I_t, the conductance g_t, the outer membrane electrical potential difference V_o and the fractional resistance fR_o of the outer membrane ($\delta V_o/\delta V_t$). The mean and range of control values of these parameters under initial short-circuit conditions in the skins studied here are shown in Table 1. In order to relate our observations to previous studies of the voltage dependence of active transport and metabolism [8, 27, 43], we first perturbed V_t symmetrically at 6-min intervals in the sequence 0, +25, −25,..., +100, −100 mV. In later experiments, in order to avoid the harmful effects of prolonged exposure to large negative transepithelial potential differences [3, 44], V_t was increased monotonically in the range $V_t > 0$ mV prior to return to short circuit, and in other experiments the frequency of alteration of V_t was increased. In some studies, following each perturbation from $V_t = 0$ mV the tissue was returned to the short-circuited state.

The nature of a typical response is shown in Fig. 1, representing an experimental record for perturbation of V_t from 0 to +100 mV and subsequent return to the short-circuit state. Except for effects of the polarity of V_t, the qualitative response was much the same for all perturbations. Thus, in association with a prompt decrease in I_t (not shown in Fig. 1), there was a prompt increase of V_o and fR_o. Although the initial changes in V_o and fR_o were quite prompt, both responses were

Table 1. Summary data of skins used in the present study under initial short-circuit conditions[a].

	I_t (μA/cm²)	g_t (mS/cm²)	V_o (mV)	fR_o (%)
Mean	56	1.46	−52	46
SD	28	0.66	21	21
Range	20 to 135	0.6 to 3.2	−93 to −33	13 to 86

[a] I_t = transepithelial current; g_t = transepithelial conductance; V_o = outer border potential; fR_o = outer border fractional resistance. $n = 17$.

Average values of g_t were higher than often observed, and we cannot rule out the possibility of edge damage in some tissues. That this was not the sole factor was indicated by the observation of high values of g_t in certain skins derived from the same batches of animals when studied in large chambers permitting minimization of edge damage. Since our emphasis was on the measurement of cellular parameters, tissues were selected on the basis of their suitability for this purpose.

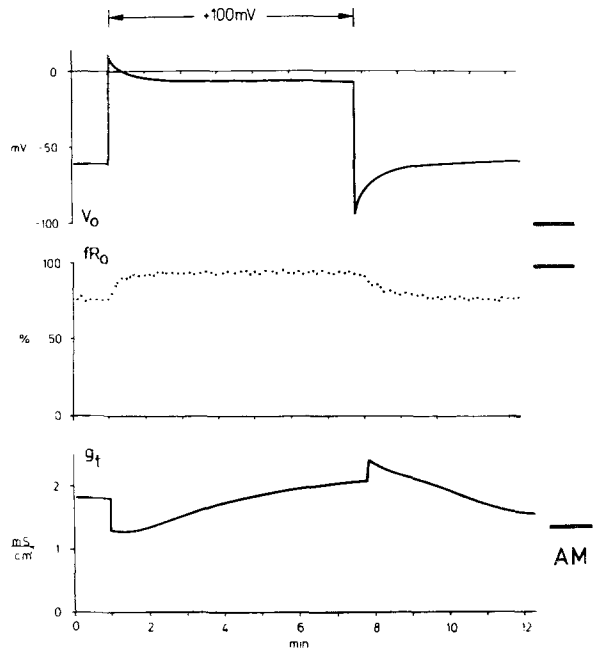

Fig. 1. Responses of V_o, fR_o and g_t to perturbation of V_t from 0 to +100 mV and subsequent return to the short-circuit state. The symbol *AM* indicates observations in the presence of 10^{-5} M amiloride. Here and elsewhere, for clarity, we have omitted transient perturbations of voltages associated with measurements of fR_o. Also omitted are values of I_t, since with marked variability of g_t it was not possible to differentiate between cellular and paracellular components

strongly time dependent; in every case there was a clear overshoot of V_o and progressive change in fR_o. Changes in g_t were more variable, and usually biphasic or multiphasic.

The details of effects on the outer membrane potential V_o and the fractional resistance fR_o are shown more clearly in Fig. 2*a* and *b*, representing the response to perturbation of V_t over an extensive range, while maintaining the microelectrode in a single cell. Figure 2*a* and *b* show tissues with high and low short-circuit values of fR_o, respectively. As is seen, responses in both tissues are qualitatively the same. Irrespective of the polarity of the change in V_t there is an overshoot of V_o, followed by a gradual relaxation of V_o to quasi-steady state levels within some 3 min or less. FR_o increased promptly with an increase in V_t and decreased promptly with decrease of V_t; in most cases fR_o changed further with time, again, however, usually reaching a quasi-steady state value within a few minutes. The fact that neither the overshoot of V_o nor the voltage dependence of fR_o is a consequence of changing the polarity of V_t is shown in Fig. 3, representing a study in which V_t was increased progressively from zero to +100 mV in steps of 25 mV.

Figure 4 shows that in general fR_o increases monotonically with increase of V_t.

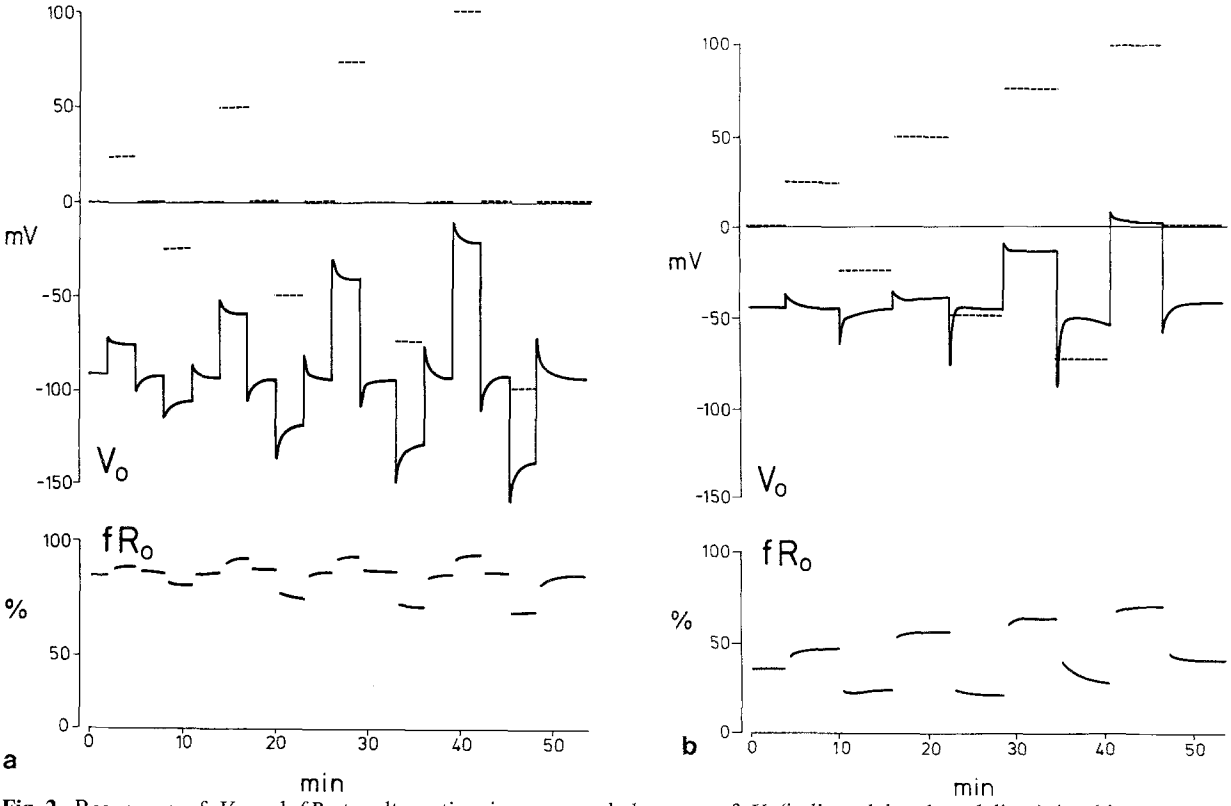

Fig. 2. Responses of V_o and fR_o to alternating increase and decrease of V_t (indicated by dotted lines) in skins with high (*a*) and low (*b*) short-circuit levels of fR_o

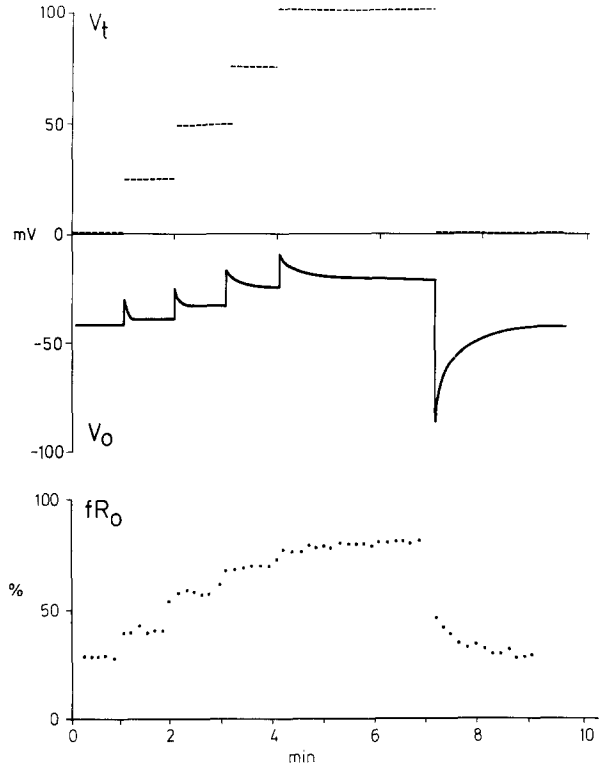

Fig. 3. Responses of V_o and fR_o to progressive increase of V_t, followed by return to the short-circuit state

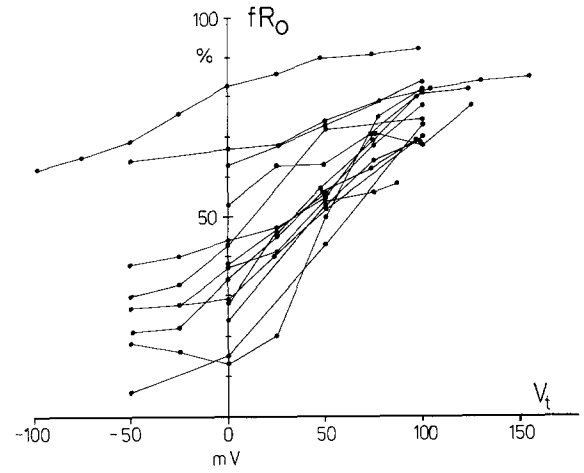

Fig. 4. Relationship between steady-state values of fR_o and V_t ($n=14$)

Time Course of Response of V_o and fR_o to Voltage Perturbation

The relaxation of the overshoot of V_o was near-exponential in all tissues (*see*, e.g., Fig. 5*a*). Half times in the various tissues ranged from 0.8 to 5 sec (mean $\pm$SD $= 2.4 \pm 1.0$ sec). Our standard protocol did not allow the accurate evaluation of the time course of fR_o. However, in five tissues, following perturbation of V_t from zero to $+50$ or $+100$ mV, fR_o was evaluated from 100 msec pulses at a frequency of 2 Hz. The approach of fR_o to its steady-state value was near exponential, with half times of 0.9 to 5 sec (*see*, e.g., Fig. 5*b*, representing the same tissue as in Fig. 5*a*). In these five tissues, the time courses of V_o and fR_o were about the same; we are not able as yet to state how generally this is the case. In one study in which V_t was perturbed sequentially to values between $+25$ and $+150$ mV the half time fell progressively from >3 to <1.5 sec, suggesting that the time constant is related inversely to the magnitude of ΔV_t. However, it is not possible to be confident about this until more precise techniques for sampling and data analysis are developed.

Localization of Voltage Dependence of fR_o

Change of fR_o on perturbation of V_t could result from changes of the conductance at either the outer cell surface (g_o) or the inner cell surface (g_i) or both. In principle, it would be possible to quantify these effects precisely, given knowledge of the transcellular conductance as well as fR_o. In practice, this was not possible in this study, since reliable values of transcellular conductance were not available. Thus, in most tissues there was pronounced voltage dependence of g_t, with progressive increase at positive V_t and decrease at negative V_t. Since these effects were often marked during periods in which fR_o remained constant, they were apparently the result of changes in passive, paracellular conductance, although we cannot, of course, rule out fortuitous proportionate changes in g_o and g_i. Under such circumstances, lacking a suitable method for evaluating the paracellular conductance repeatedly during the course of an experiment, it is not possible to know precisely the time course of the cellular conductance or its components g_o and g_i.

Despite this limitation, it appeared possible to localize the predominant effect on the cellular conductance by qualitative studies during the very early period immediately following perturbation of V_t. During this period, before the passage of sufficient time for the development of appreciable change of paracellular conductance, if the predominant effect on cellular conductance is at the outer cell membrane, changes in g_t should be in an opposite sense to changes in fR_o, whereas if the predominant effect is at the inner cell membrane (or the basolateral interstitial space), the converse should be the case. Consideration of Fig. 6 in conjunction with Figs. 1–4 suggests that the concur-

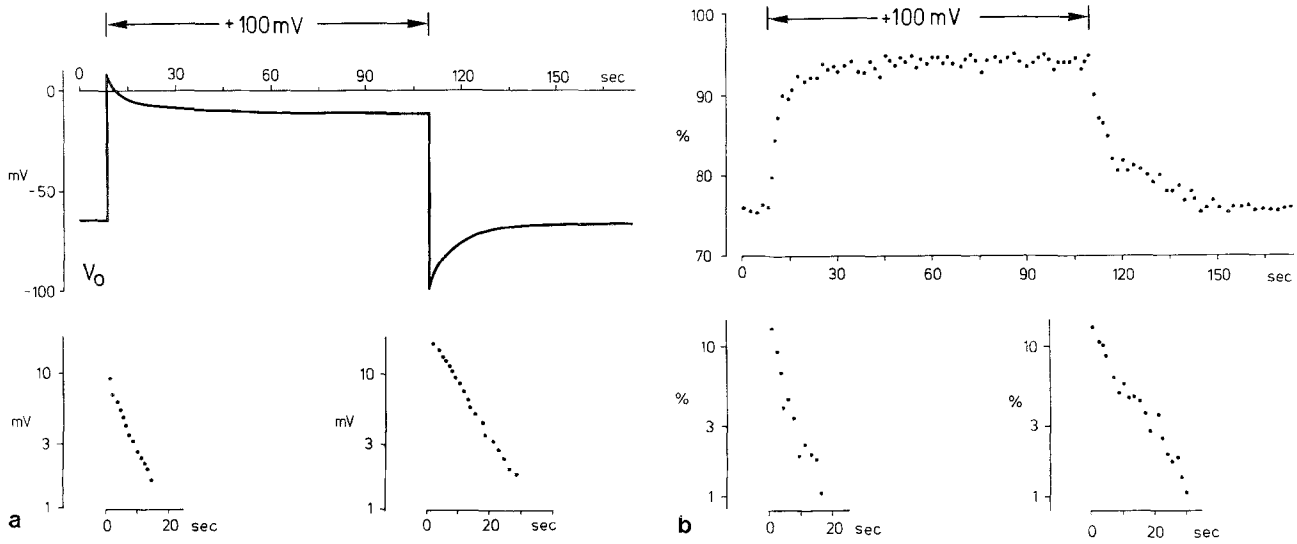

Fig. 5. Time course of response of V_o (*a*) and fR_o (*b*) to perturbation of V_t from 0 to +100 mV and subsequent return to the short-circuit state. The semilogarithmic plots show that the half times for response of V_o and fR_o to hyperpolarization were 5.0 and 3.8 sec, respectively, and for depolarization were 8.6 and 8.0 sec, respectively

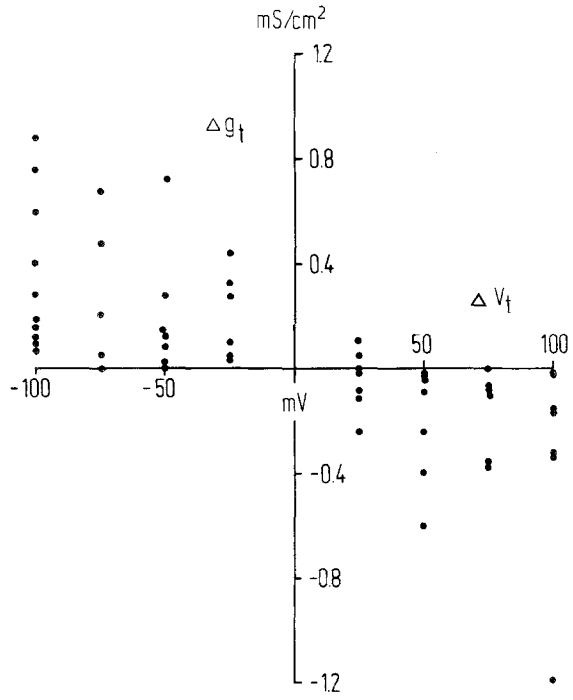

Fig. 6. Early changes in g_t induced by increase or decrease of V_t. Studies involving large negative values of V_t were excluded

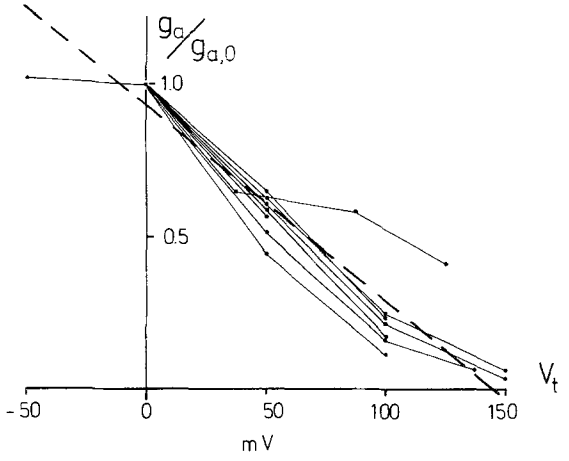

Fig. 7. Relationship between normalized g_a and V_t ($n=7$). Values of g_a are normalized with respect to the initial value at short circuit ($g_{a,0}$) in the same tissue. The dashed line represents the mean of the linear least square relationships of the individual experiments.

rent changes in g_t and fR_o were related inversely, as is consistent with a predominant effect at the outer cell surface.

Support for this interpretation was provided in an additional group of studies by the determination of amiloride-sensitive conductance at different settings of V_t. As is shown in Fig. 7, the magnitude of the amiloride-sensitive conductance was related inversely to V_t, again indicating an inverse relationship between outer membrane conductance and V_t.

Further substantiation for the predominance of the effect at the outer membrane was obtained in three studies in which internal parameters were measured in conjunction with measurement of transepithelial conductance. Figure 8 compares the effects of amiloride on V_o, fR_o and g_t in a cell at short circuit (Fig. 8*a*) and in the same cell ~50 sec after clamping V_t at +100 mV (Fig. 8*b*). As is seen, at 100 mV the effects of amiloride on all three parameters were greatly diminished. The reduced effectiveness of amiloride upon hyperpo-

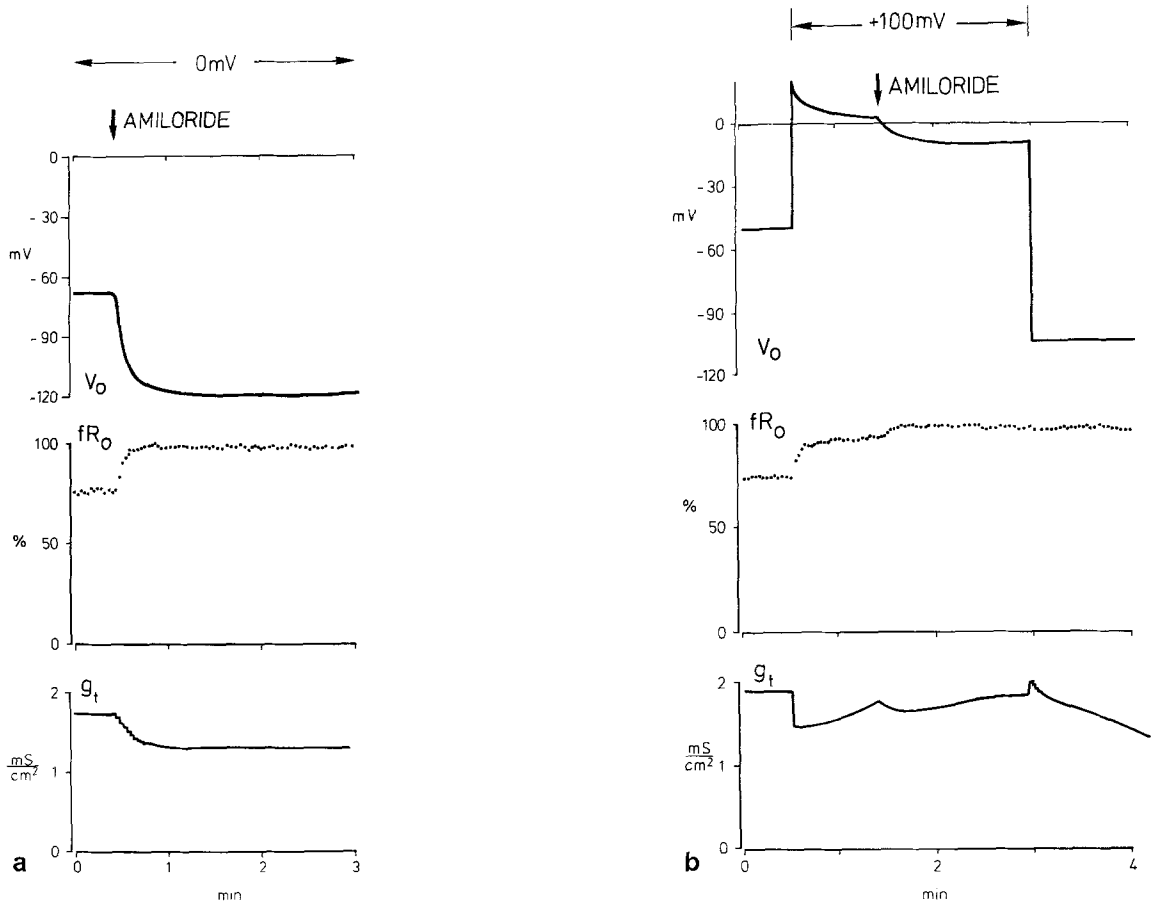

Fig. 8. Effect of 10^{-5} M amiloride on V_o, fR_o and g_t in a cell initially at short circuit (*a*) and after clamping V_t at +100 mV (*b*)

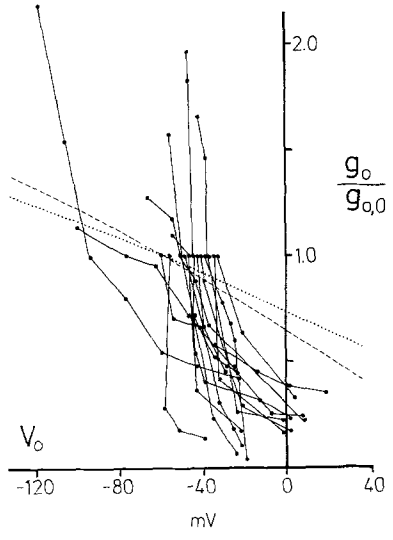

Fig. 9. Relationship between normalized g_o and V_o ($n=14$). Values of g_o are normalized with respect to the initial value at short circuit in the same tissue by means of calculations based on values of fR_o, assuming g_i independent of V_t (*see* text). The dotted and dashed lines represent theoretical relationships predicted from the constant field equation (*see* text)

larization seems unlikely to result from a change in the mechanism of binding, since in all three tissues studied in this way the value of fR_o in the presence of amiloride was independent of V_t.

Relationship of g_o to V_o; Constant Field Equation

In speculating as to factors influencing g_o we considered first its possible dependence on V_o. Clearly, in order to examine the effects of V_t systematically it would be desirable to have precise values of g_o and g_i. For reasons discussed above, this was not possible. However, assuming that g_i is unaffected by V_t, it is possible even without knowledge of the paracellular conductance to quantify the effect of V_t on g_o by expressing values of g_o at $V_t \neq 0$ relative to those in the short-circuit state ($g_{o,\,o}$). This ratio is evaluated from the following identity:

$$g_o/g_{o,\,o} = (1 - fR_o) \cdot fR_{o,\,o} / (1 - fR_{o,\,o}) \cdot fR_o .$$

Figure 9 shows the dependence of normalized values of g_o on V_o. The dotted line shows the slope

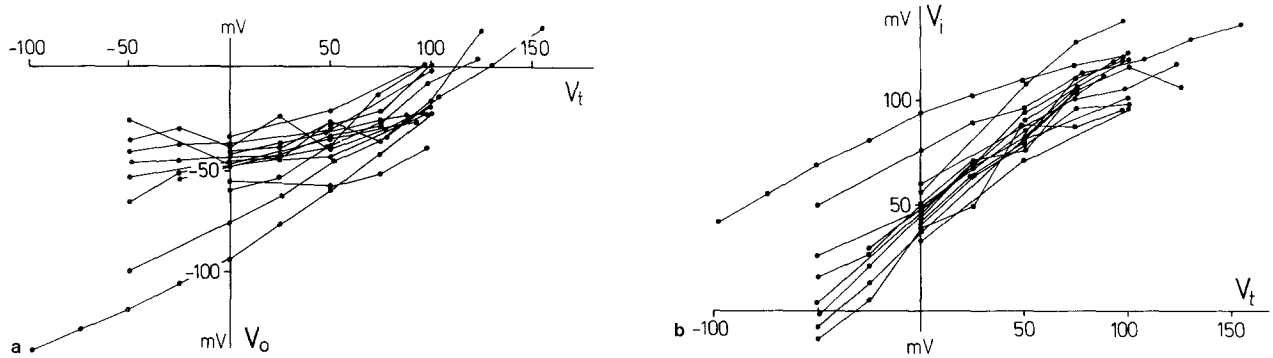

Fig. 10. Relationship between steady-state values of V_o and V_t (*a*) and V_i and V_t (*b*) ($n=14$)

that would be predicted from the constant field equation [15, 18, 25], assuming constancy of the internal Na^+ concentration at 15 mM, and the dashed line that which would be seen with a decline of Na^+ concentration from 15 to 5 mM as V_o increases from -65 to $+15$ mV. Evidently, the slope observed experimentally is much steeper than that predicted by the constant field equation on either basis. (These comparisons were made assuming that the outer cell membrane conductance is attributable only to Na^+. Given plausible cellular concentrations of Cl^- and K^+, finite mobility of either of these ions would reduce the negative slope of the theoretical relationship to still lower values.)[1]

Steady-State Voltage Relationships

The steady-state dependencies of V_o and V_i on V_t are shown in Fig. 10*a* and *b*, representing the composite results in all tissues. One significant finding is that in most tissues the slope of V_o versus V_t is rather flat, so that at $V_t=+100$ mV V_o was generally negative. This is particularly impressive in view of the positive correlation of fR_o with V_t (Fig. 4), which might have been expected to increase the voltage dependence of V_o. This observation points to the importance of the distinction between steady-state relationships, as in the observations of Fig. 10*a* and *b*, and short-term voltage dependencies, as are involved in the estimation of fR_o. Also noteworthy was suggestive linearity of the dependence of V_i on V_t.

[1] Some workers have suggested that g_i increases with increasing transcellular current, but this conclusion appears to derive from comparisons between different tissues [16, 40]. To the best of our knowledge no one has demonstrated that increasing transcellular current in a given tissue is regularly associated with increase of g_i. If this were the case, it would be necessary to modify the calculation of $g_o/g_{o,\,o}$ by inclusion of the factor $(g_i/g_{i,\,o})$, which would result in still greater deviation from the relationships predicted by the constant field equation.

Discussion

Despite the fact that current-voltage relationships are widely employed to interpret epithelial function, relatively little is known concerning the effects of transepithelial potential on the intracellular state and apical and basolateral membrane function. Lacking such information, the choice of a protocol to evaluate the effect of an experimental variable is to some extent necessarily arbitrary. Thus, it is commonly tacitly assumed that since brief, small changes of V_t can have only little effect on cellular ion and water content, such perturbations permit the evaluation of the membrane parameters of the undisturbed system. Others consider, however, that valid inferences concerning function require extended periods of perturbation of transepithelial forces, in order to permit the establishment of a steady state.

In practice, neither of these approaches is without a potential drawback. With brief perturbations of transepithelial potential it cannot be assumed that transcellular current is attributable only to the active transport process of interest. In the frog skin or toad urinary bladder, for example, where steady-state transcellular current is attributable very largely to sodium, for some period following perturbation of V_t sodium flow may be nonconservative, with an appreciable fraction of the current across the basolateral membrane representing flow of potassium and/or chloride [12, 21, 31, 48]. With the passage of time, transepithelial sodium flow is again conservative [47], but prolonged perturbations of V_t may affect the membrane so as to alter the very parameters under study. Thus in the frog skin large perturbations of V_t for 15 min or longer produce a "memory effect," such that subsequent values of the short-circuit current and the associated rate of oxygen consumption differ appreciably from those measured initially [43]. Inward current flow for extended periods has been shown to

induce swelling of the stratum granulosum in the frog skin [44] and of granular cells in the toad bladder [3].

The present study provides several observations relevant to the above considerations. Promptly following perturbation of V_t, a series of dynamic changes occurs. Given the nature of our recording system and data processing techniques, it is clear that the events reported here occur after the completion of capacitive effects. Promptly after change of V_t there was overshoot of the outer membrane potential, followed by relaxation to near steady-state levels, usually within some 1 to 3 min. This is consistent with earlier observations of amiloride-sensitive current and oxidative metabolism in anuran epithelia [27, 38, 43]. The early changes of potentials were associated with prompt change of the total conductance and the fractional membrane resistance. In our standard protocols marked effects on fR_o were observed as rapidly as we made the measurements; in most cases there was a moderate further change with maintenance of the new setting of the clamping potential.

Although ignorance of the time course of paracellular conductance and intracellular activity patterns prevents complete analysis of the factors influencing transport at the two limiting membranes, several observations deserve comment. One striking finding was the overshoot of V_o observed regularly after perturbation of V_t. Although we are unable as yet completely to explain this effect, a simple equivalent circuit analysis suggests that it may be a consequence of the nature of the voltage dependence of fR_o[2]. Thus in periods of conservative current flow, when outer membrane and transcellular current are equal,

$$I_a=(E_o-V_o)/R_o=(E_a-V_t)/R_a$$

where E_o and E_a are effective electromotive forces for the outer membrane and cell, respectively, and R_o and R_a $(=1/g_a)$ are the corresponding resistances. Rearrangement then gives

$$V_o=E_o-fR_o\cdot(E_a-V_t).$$

On perturbing V_t, assuming initial constancy of E_o and E_a, we have

$$dV_o/dt=fR_o\cdot(dV_t/dt)-(E_a-V_t)\cdot(dfR_o/dt).$$

For a step change in V_t, initially dV_t/dt is very large, so that dV_o/dt and dV_t/dt are of the same sign. An instant later, when $dV_t/dt=0$, dV_o/dt will be proportional to dfR_o/dt and for positive transcellular current $I_a=(E_a-V_t)/R_a$, opposite in sign. Furthermore, if V_o and fR_o are exponential functions of time, their time constants must be equal. This is consistent with our findings, but more precise measurements are needed.

Another impressive finding was the magnitude of effects of V_t on membrane conductance. Various workers have demonstrated that in some circumstances current-voltage relationships in frog skins and toad bladders are highly non-linear [e.g. 6, 10, 17, 23]. However, others have found that with brief perturbation of V_t in appropriate ranges the relationship is approximately linear [9, 10, 39, 45]. Furthermore, in the quasi-steady states achieved some 4–6 min following symmetric perturbation of potential, the dependence of both transcellular current and suprabasal oxygen consumption on V_t is linear over a range of at least ± 80 mV [8, 27]. Presuming that such linear dependencies of cellular parameters exist in the tissues examined by us (despite our inability to demonstrate these owing to voltage-dependence of paracellular conductance), it might have been expected that membrane conductance and thus fR_o would remain constant over a large range of transepithelial potential. As is shown in Fig. 4, this was far from the case. The dependence of fR_o on potential was marked, and indeed usually most marked, in the physiological range of positive V_t.

In analyzing the inverse relationship between g_o and V_o in the light of observations of others, it is perhaps surprising to find a clearly stronger voltage dependence than would be predicted from the constant field equation [15, 18, 25]. It must be emphasized, however, that here we are discussing the dependence of g_o on steady-state settings of V_t. Others who have inferred constant field behavior at the outer membrane have considered $I-V$ relationships in studies with rapid alterations of clamping potential, as well as other differences from our protocol. For example, Fuchs, Hviid Larsen and Lindemann, in their studies in frog skins, used a staircase-shaped voltage signal with between 6 and 30 steps of 20 msec duration [17]. Using sulphate solution with external sodium concentrations between 5–50 mM and high internal potassium concentrations, their $I-V$ relationships

[2] Although in comprehensive analyses of equivalent circuit theory, it is important to relate slope and chord conductances [21], useful qualitative insights may be derived from consideration of slope conductances as measured by our techniques. Accordingly, the equivalent circuit relationships considered by us are merely rearrangements of the fundamental definitions of the "effective electromotive forces," *viz.* $E_o\equiv I_aR_o+V_o$; $E_i\equiv I_aR_i+V_i$; $E_a\equiv I_aR_a+V_t$. For later more precise analysis it will, of course, be important to determine the time dependence of the response of these E's to perturbations of V_t. This would help to elucidate the extent to which it is possible to reason about steady-state behavior on the basis of parameters derived from transients [48].

could be fit to the constant field equation over a voltage range of 0 to +50 mV (our polarity convention). Presuming near-complete depolarization of the inner cell membrane, as suggested by Fuchs et al., this represents a range of V_o values of 0 to +50 mV, as compared with the range of about −140 to +15 mV examined in the present study. Clearly, because of these several differences in protocol, our two studies are not comparable. Nevertheless, we would suggest that in interpreting the nature of $I-V$ relationships due attention should be given to the possible importance of complex time-dependent voltage dependencies of g_o, as observed in the present study.

In an interesting recent article Gordon has suggested that the conductance in the toad urinary bladder may be associated only with the paracellular pathway for ions [19]. The very rapid marked changes in g_t as well as fR_o on voltage perturbation or application of amiloride lead us to believe that this is unlikely to be the case for the frog skins studied by us. However, a definitive conclusion on this point must await more precise temporal observations than are presently available.

Our finding of an inverse correlation between V_t and amiloride-sensitive conductance is consistent with Cuthbert and Shum's demonstration of an inverse relationship between transepithelial potential and displaceable amiloride binding, presumably reflecting effects on the number of sodium translocation sites [11]. Although further insights into the mechanism of effects on membrane conductance must await their exact quantification with adequate temporal resolution, as well as knowledge of the influence of V_t on cell ionic activities, one obvious possibility to be considered is that g_o might be influenced by the magnitude of V_o. As is seen in Fig. 9, assuming constancy of g_i, an inverse relationship was found between steady-state values of g_o and V_o. It is not possible to state, however, that the effect on V_o was responsible for that on g_o. Moreover, under some circumstances it is not possible to demonstrate an inverse correlation between g_o and V_o. Increase of V_o induced by serosal addition of Ba^{++} or K^+, serosal hypertonicity, or serosal reduction of Na^+ is associated with either no change or increase of g_o [34, 36, W. Nagel, *unpublished results*). Also, in a study of Higgins, Gebler and Frömter in open-circuited *Necturus* urinary bladders nearly uniform high values of fR_o were found in tissues with values of V_o ranging from about −50 to +50 mV [24, Fig. 9]. Transcellular current in these studies was rather low, as will be shown below. This raises the possibility that V_t may influence g_o and fR_o by its effects on transcellular current. Still another possibility is that the effects of voltage perturbation are indirect, consequent to influences on, for example, cellular-extracellular distribution of Na^+, Ca^{++}, H^+, and/or K^+, [3, 20, 42, 44, 46]. These and other possibilities remain to be investigated.

The demonstration of high values of fR_o is critically dependent on proper sealing of the electrode in the cell membrane [22, 24, 30, 33, 37]. With careful attention to technical detail, Helman and Fisher demonstrated values of fR_o averaging 75.6% in untreated frog skins [22]. As in previous studies of Helman and his associates [21, 23], the $I-V$ relationships characteristically showed three regions of linearity, with discrete changes in slope ("break points"). Although no discrete change of fR_o with V_t was noted by us, again there need be no inconsistency, since the conditions of the studies were different and, as they state, despite their usual finding of a discrete break point at E_1, there is "undoubtedly a curvilinearity in the transition zones."

Also of interest are the experiments of Higgins et al. and Frömter and Gebler in *Necturus* urinary bladders, punctured from the serosal surface so as to eliminate damage of the high-resistance mucosal membrane [16, 24]. The findings differed from ours in their higher average value of fR_o, as well as apparent lack of voltage dependence of both fR_o and V_i. Despite variation of V_t over a range of about 30 to 140 mV, fR_o was nearly constant at a high value of 93 ± 3.2 (SD)%, and V_i varied only from about 75 to 90 mV (*see* their Fig. 9). Again, however, it is possible that the differences between the findings of the two studies are not substantive, but result from differences in protocols. In Higgins et al.'s and Frömter and Gebler's studies of open-circuited tissues, in which different values of V_t existed only in different tissues, transcellular current was necessarily small at all values of open-circuit potential, owing to the high values of leak resistance in their system. (At open circuit the transcellular "active" current is equal in magnitude to the oppositely directed transepithelial "passive" current.) In eight satisfactory serosal cell punctures in five bladders values of parallel shunt resistance ranged from 18 to 64 $k\Omega\,cm^2$, open circuit potential ranged from 33 to 126 mV, and the circulating current at open circuit varied only from 0.7 to 3.8 $\mu A/cm^2$ (E. Frömter, *personal communication*). In contrast, in the present studies values of transcellular current averaged 56 $\mu A/cm^2$ in the short-circuited state and would have fallen to about half this value on raising V_t to 70 mV, about half the value of E_{Na}. Further studies are

required to determine whether the level of transcellular current, rather than the value of V_t, *per se*, is the important determinant of fR_o. Whether or not this is so, it appears possible that the small value of transcellular current may account for the near constancy of V_i in Higgins et al.'s and Frömter and Gebler's studies. This may be appreciated by considering the equivalent circuit relationship: $V_i = E_i - I_a \cdot R_i$, where E_i represents the effective electromotive force across the inner (basolateral) cell membrane and R_i is the electrical resistance across this surface. This shows that in tissues in which $I_a \cdot R_i$ is near zero, V_i can vary only to the extent that there is variation in E_i. In the above cited serosal cell punctures $I_a \cdot R_i$ ranged from 1.8 to 8.7 mV and V_i ranged from 74 to 90 mV (E. Frömter, *personal communication*), indicating near constancy of E_i. The extent of variability in E_i in different tissues under diverse circumstances remains, however, to be determined precisely[3].

An important consideration in the analysis of the energetics of active sodium transport is evaluation of the rate of work performed by the basolateral sodium pump. Thus Leaf, Chu and Schultz have addressed the question of whether changing the transepithelial potential significantly affects the work done per sodium ion transported [29]. For this purpose they have drawn on the above-discussed studies of Higgins et al. in which, as mentioned, the electrical resistance of the apical plasma membrane was about 13 times greater than that of the basolateral membrane and the basolateral potential was near constant at about 90 mV. Applying these findings to the toad urinary bladder, Leaf et al. calculated that on hyperpolarization by 70 mV, 65 mV of the transepithelial potential will be partitioned to the apical plasma membrane, so that V_o will change from -90 to -25 mV, while V_i will change only from $+90$ to $+95$ mV. Combining these values with measured values of the rate of transepithelial Na transport in the short circuit and hyperpolarized steady states, they calculated a decrease of the cellular sodium concentration from 12.0 to 11.6 mM on hyperpolarization. Thus it appeared that the work of transporting a sodium ion across the basolateral plasma membrane increased only 1.5% as a result of the slight increase in the concentration gradient and 5.6% as a result of the increase in the electrical gradient. The small magnitude of these changes was believed to be consistent with the earlier conclusion of Leaf and his colleagues that the metabolic cost of transporting a sodium ion across the bladder was undetectably different in steady states at short circuit or at $V_t = +70$ mV as judged from measurements of CO_2 production [26].

As can be seen, the findings of the present study differ significantly from those which would be predicted from the above calculations (*see* Fig. 10*a* and *b*). According to our findings, changes of V_t of the magnitudes considered above would result in quite appreciable changes in the electrochemical work per sodium ion transported. In attempting to explain this discrepancy, in addition to obvious species differences at least three points appear worth consideration: (i) The values of fR_o observed in Higgins et al.'s studies at open circuit were appreciably higher than most of those observed by us. (ii) The membrane resistance ratios in these studies, as with the fR_o's of the present study, were deduced from voltage divider ratios following a short perturbation of V_t (in their case 1–2 sec). To the extent that transient flows of K^+ and Cl^- contribute to basolateral current during voltage perturbations the ratio of electrical resistances R_o/R_i will overestimate the corresponding ratio of resistances to Na flow at the two plasma membranes, $R_{o,\,Na}/R_{i,\,Na}$. They will therefore lead to underestimation of the magnitude of the change in the driving force for Na flow across the basolateral membrane in the hyperpolarized steady state, when transepithelial Na flow will again be conservative and equivalent to the transcellular current flow [48]. (iii) In the present study fR_o was not constant, but increased with V_t, and for reasons discussed above it appears that this change is for the greater part attributable to decrease of apical Na conductance. Under such circumstances, a given decrease of transcellular current with hyperpolarization will be associated with a smaller decrease in the driving force across the apical membrane $(E_o - V_o)$ and thus a larger decrease in that across the basolateral membrane $(E_i - V_i)$ than would be the case with a constant resistance ratio. This can be seen by considering that since on the time scale of the present experiments transcellular current is conservative,

$$\delta I_a = \delta[g_o \cdot (E_o - V_o)] = \delta[g_i \cdot (E_i - V_i)].$$

Evidently, if g_o and g_i are constant the ratio of the changes in basolateral and apical driving forces $\delta(E_i - V_i)/\delta(E_o - V_o)$ will be given by g_o/g_i. If, how-

[3] With respect to the contribution of the Na^+ pump to E_i, an increase in "apparent E_{Na}" with hyperpolarization has been reported by Canessa et al. [7], Feig et al. [14], and Wolff and Essig [48]. However, values of apparent E_{Na} derived from short-term conductance measurements as in the above studies cannot be unequivocally related to "true" values of E_{Na}, pending determination of the contribution of flows other than that of sodium to current following transient perturbations of potentials [48].

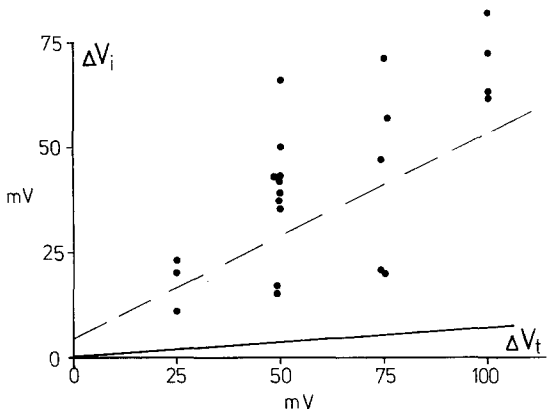

Fig. 11. Changes in steady-state value of V_i induced by increase of V_t (22 observations in 12 tissues). The dashed line represents the mean relationship which would be predicted on the basis of constancy of fR_o at the level measured initially at short circuit in each tissue. The solid line represents the relationship which would be predicted assuming $fR_o = 93\%$, as reported by Higgins et al. [24]

ever, g_o decreases while g_i remains constant on hyperpolarization, the same decrease in I_a and basolateral driving force as in the first case will be associated with a smaller decrease in apical driving force. Therefore, the relative change in the basolateral driving force will be larger.[4]

The significance of the above factors is suggested by Fig. 11, which compares our experimentally observed changes in V_i on voltage clamping with the changes in basolateral driving force predicted on the basis of the assumption that fR_o is constant and comprises only resistance to sodium flow. Here the dashed line represents predictions based on our values at short circuit, and the solid line represents predictions based on the value of 93% observed in the studies of Higgins et al. As is seen, in most cases V_i changes to a greater extent than predicted on either basis for the total driving force. To the extent that there may also be diminution of cell Na activity with hyperpolarization, with increase in the adverse chemical potential gradient across the basal lateral membrane, the discrepancy will be somewhat greater. (Of course these calculations do not take into account possible changes in E_{Na}; the magnitude of such a change is at present unknown. *See* footnote 3.)

The protocols of potential perturbation in this study were similar to others shown previously to result in quasi-steady states with near-linear voltage dependencies of transepithelial active sodium transport and oxidative metabolism in toad urinary bladders and frog skins (*R. pipiens*) [8, 27, 28, 43, 48]. In most of the skins studied by us here (*R. temporaria* and *R. esculenta*) there were pronounced effects of perturbation of V_t on apical conductance, with variable effects on steady-state V_o; on the other hand, a suggestively near-linear relationship was observed between V_i and V_t. Presuming linearity of the cellular system, as in earlier studies, a question of fundamental importance is the nature of the regulatory mechanisms resulting in such linearity. Further insight into this issue must await combined precise measurements of net sodium flow and both chemical and electrical potential differences across both apical and basolateral plasma membranes.

[4] With sufficiently great decrease in g_o on hyperpolarization, it is in principle possible that there might be no decrease or even an increase in $(E_o - V_o)$ in association with the decrease in transcellular current. This effect might possibly account for the decrease in V_o observed with increase in V_t in several instances in Fig. 10*a*. The same consideration points to the need for caution in inferring values of E_{Na} from steady-state current voltage relationships.

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