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Letters to the Editor

Time-Dependent Phenomena in Voltage-Clamped Epithelia

Two recent publications in *J. Membrane Biol.* (Schultz et al., 1984*a*; DeLong & Civan, 1984) deal with time-dependent phenomena in voltage-clamped epithelia and their influence on the current-voltage relations of cell membranes. In both articles the authors present the view that these relations are not distorted significantly by capacitative effects.

In a study of the current-voltage relations of the cell membranes of *Necturus* urinary bladder, Schultz et al. (1984*a*) found a shift in the basolateral *I-V* relation between 20 and 90 msec after onset of the transepithelial clamp pulse. Five arguments were presented in support of the view that this temporal variation in the *I-V* curve is not due entirely to capacitative effects. A later *Erratum* (Schultz et al., 1984*b*) pointed out an error in the original Eq. (A4) describing the time-dependence of the apical membrane potential. For this reason, three of their arguments (2, 3, 4) were acknowledged to be incorrect. However, on the basis of the remaining two arguments, they persisted in their view that the observed time-dependence of the *I-V* relation cannot be explained entirely by capacitative transients. The first argument (p. 262) is based on the observation that the *I-V* relationship of the apical membrane fits the same constant field equation at 20 and 90 msec while the basolateral *I-V* relation changes during this time period. From this they concluded that the observed distortion of the basolateral *I-V* relation is of noncapacitive origin since capacitative transients should distort *both* membranes alike. However, close examination of the expressions for cell current, $I^c(t)$, and apical voltage $\psi^{mc}(t)$, indicates that this need not be the case. From their Eq. (A1) and the correct expression for $\psi^{mc}(t)$ (Schultz et al., 1984*b*, Eq. (A4)), it can be shown that, in the linear approximation,

$$\frac{\Delta I^c(t)}{\Delta\psi^{mc}(t)} = -\frac{1}{r^m} \frac{\left(\frac{r^m}{r^m + r^s}\right) + \left(1 - \frac{\tau^m}{\tau}\right)\left(\frac{C^s}{C^m + C^s} - \frac{r^m}{r^m + r^s}\right) e^{-t/\tau}}{\left(\frac{r^m}{r^m + r^s}\right) + \left(\frac{C^s}{C^m + C^s} - \frac{r^m}{r^m + r^s}\right) e^{-t/\tau}} \quad (1)$$

where $\tau^m = r^m C^m$ and the Δ's are changes from the initial state (*see also* DeLong & Civan, 1984, Eqs. (17A) and (19A)). Thus if $\tau^m \ll \tau$, the above expression would simplify to

$$\frac{\Delta I^c(t)}{\Delta\psi^{mc}(t)} \simeq -\frac{1}{r^m} \quad (2)$$

and the relation between apical (cell) current and voltage at any time t after the onset of the pulse would be determined by r^m alone. Present knowledge of membrane time constants is highly inexact. However, assuming for discussion values of electrical resistances and capacitances in tight epithelia reported here (Schultz et al., 1984*a*, p. 263), $\tau^m \ll \tau$. If so, *even during dissipation of capacitative transients,* the *I-V* relation of the apical (but not the basolateral) membrane would be expected to remain undistorted although both $I^c(t)$ and $\psi^{mc}(t)$ change with time, as was observed by Schultz et al. (1984*a*) and DeLong and Civan (1984, Fig. 11).

The remaining argument presented by Schultz et al. (1984*b*) refers to their estimate of τ (p. 263). Using the value of r^s calculated from measurements at 20 msec, which implicitly assumes that the capacitative transient had almost dissipated at this time, they calculated $\tau \simeq 15$ msec. If, on the contrary, one uses the value at 90 msec (calculated from their Fig. 5 and Table) τ increases to about 33 msec. Therefore their argument is tautological and does not support their view.

The study of DeLong and Civan (1984) concerned primarily the *I-V* relation of the apical membrane of split frog skin. These authors found that the response of $\psi^{mc}(t)$ to clamping of the transepithelial potential exhibited an initial fast component with a time constant of between 1.1 and 1.6 msec and a later relaxation (which they recorded for only some 30 msec) towards the original steady-state value of ψ^{mc}. It was claimed that the initial deflection of $\psi^{mc}(t)$ represents the capacitative transient whereas the later relaxation (which they did not analyze) was attributed to other factors, e.g. changes in fractional resistance and/or polarization. Without specific information on the electronic circuitry of their voltage-clamp and microelectrode recording system (e.g., settling time of the clamp amplifier, frequency response of the unshielded microelectrode) we are forced to suspect that the fast initial transient may well reflect the bandwidth limitation of their instrumentation system. Furthermore, the authors present (p. 28) two arguments that the later relaxations do not reflect capacitative effects: The first contrasts the time course of ψ^{mc} following the first few msec in chloride and sulfate Ringer's solutions. This is an obscure argument and deals with a time period that may well be inside the range considered above. The second argument rests on the abolition by amiloride of the slow time-dependent reduction in $|\psi^{mc} - \psi_o^{mc}|$, whereas on the basis of a basolateral membrane conductance far in excess of that of the apical membrane the authors would expect amiloride to have little or no effect on the time constant (their Eq. (11A)). However, the data presented do not demonstrate an effect on τ. An alternative reasonable possibility is that $C^s \gg C^m$ (*see* Schultz et al., 1984*a*, p. 263), so that $C^s/(C^m + C^s) \simeq 1$; in the presence of amiloride r^m becomes $\gg r^s$, so that $r^m/(r^m + r^s)$ also approximates 1. Thus the amplitude of the exponential term in $\Delta\psi^{mc}(t)$, given by $C^s/(C^m + C^s) - r^m/(r^m + r^s)$ (Schultz et al., 1984*b*, Eq. (A4′); DeLong & Civan, 1984, Eq. (17A)), would approach zero.

Under these circumstances the time-dependence of capacitative relaxation of $\Delta\psi^{mc}$ would not be apparent in the presence of amiloride, irrespective of the value of τ.

In summary, the publications of Schultz et al. (1984*a*) and DeLong and Civan (1984) do not provide any acceptable evidence against capacitative transients as the origin of the observed relaxation of intracellular potentials after transepithelial voltage perturbation.

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Reply to: Time-Dependent Phenomena in Voltage-Clamped Epithelia

The aim of our recently published study (DeLong & Civan, 1984) was to address two questions: (i) can the relationship between membrane potential (ψ^{mc}) and sodium current (I_{Na}) across the apical membrane of frog skin epithelium be fit by the constant field equation, and (ii) can application of high serosal K^+ permit the rigorous determination of the $I_{Na} - \psi^{mc}$ relationship across frog skin epithelium solely by transepithelial measurements. This aim was explicitly stated on p. 36 of that study. The second of the two questions was largely answered by measuring the apical membrane potential with the transepithelial potential ψ^{ms} clamped to zero, and did not require the application of trains of voltage pulses. The first question could be addressed by applying series of brief voltage pulses, because the apical membrane potential changed very little during the latter 27–30 msec of each 31-msec voltage step. Whether the slight time-dependent changes in ψ^{mc} during these 27–30 msec reflected polarization effects (as we suggested) or capacitative effects (as suggested by Drs. Garcia-Diaz and Essig) is not critical to the analysis. The central observation was that such changes were sufficiently small not to obscure the excellent fit of the Goldman equation to the $I_{Na} - \psi^{mc}$ relationship, even over a range of very early times. The great merit of carrying out the measurements at such early times was to minimize the contributions of complex time-dependent phenomena well-documented to appear after prolonged voltage steps (Weinstein et al., 1980).

In our published study, Fig. 3 presents the responses of the apical membrane potentials to transepithelial polarizations of ±100 mV. Three phases can be distinguished. In four of the five preparations, phase I of the response was a brief spike (<1 msec), overshooting the steady-state response in two cases. In two of the preparations (Figs. 3*d* and *e*), a phase II was noted in which the spike was followed by a slower increase to the peak polarization response over another 2–3 msec. In almost all of the preparations, the responses also displayed a phase III, in which the apical membrane potential decayed slightly towards the prepulse value. The rates of these decays were variable. For the depolarizing pulse of Fig. 3*c*, no decay was noted, whatsoever; in each case, however, the decay constituted only a small fraction of the total response of ψ^{mc} to step changes in ψ^{ms}.

Our interpretation has been that the phase I response reflects the effects of surge currents from the voltage clamp and membrane capacitative effects. Although not explicitly discussed in the manuscript, the appearance of an overshoot in ψ^{mc} during phase I surely reflects a transfer of energy from the active element (the voltage clamp) operating in an underdamped mode in the experiment of Fig. 2. We have interpreted phase II to reflect the capacitative effects of the membranes, at least in those tissues bathed with sulfate. If this interpretation is correct, we would conclude that the time constant of the apical membrane (τ_a) is greater than that (τ_b) of the basolateral membrane (Eq. (17A), Fig. 4*B*), consistent with the (unmentioned) observation that amiloride did not alter phase II of the sulfate-bathed epithelia. In our interpretation, phase III was considered to reflect changes in apical fractional resistance and/or polarization within the tissue. This interpretation is consistent with the observation that amiloride both abolishes the phase III response and the time-dependent transepithelial electrophysiological phenomena which occur over periods of seconds, and which cannot reflect simple membrane capacitative effects (Weinstein et al., 1980).

In essence, the Letter to the Editor has suggested that phases I and II could both reflect the time responses of the voltage clamp and of the recording micropipette. In contrast, phase III would reflect the membrane capacitative effects. If so, this would conform to trajectory 3 (Fig. 4*B*) of our paper, suggesting that $\tau_a < \tau_b$. This interpretation would also be consistent with the data obtained after addition of amiloride.

In an effort to examine these two interpretations further, we have conducted two types of simulation studies. In the first, an experiment was conducted in which a Millipore filter (0.48 μm pore size) simulated the apical plasma membrane and a perforated Parafilm membrane simulated the basolateral membrane. Ringer's solutions were placed in the "serosal," "cellular" and "mucosal" phases. A typical micropipette was introduced into the "cellular phase." In addition, in collaboration with Mrs. Kim Peterson-Yantorno, a second set of seven simulations was conducted with six split frog skins. Typical tissues and micropipettes were used, but with the mucosal and serosal leads reversed. In contrast to a similar control conducted previously, a wide range of micropipette tips was used, whose resistances varied from 38 to 262 MΩ (with a mean ± SE of 154 ± 33 MΩ); with each of these micropipettes, technically satisfactory impalements were obtained. As is now customary in our laboratory, cells were impaled across their basolateral membranes. However, with the transepithelial leads reversed (grounding the mucosal voltage lead), the transepithelial potential could now be measured both with the standard agar leads, and with the exploring micropipettes in the serosal bath; comparison of the two measurements of ψ^{ms} permitted assessment of the response times of the micropi-

pettes. A response time T was defined as the time (following a transepithelial step polarization to ±100 mV) required for ψ^{mc} to settle to within 5 mV of its steady-state level. Defined in these terms, T was measured to be 0.2–2.3 msec, with a mean ± SE of 1 ± 0.2 for the eight experiments. As anticipated, the lower values of T characterized the micropipettes with lower resistances; however, satisfactory impalements (including responses to pulse trains) were attained with all micropipettes used.

We regard the data obtained with the simulations as consistent with either interpretation. The suggestion that phase II reflected the response times of the micropipettes appears entirely plausible. However, if the later phase III did, in fact, reflect a capacitative event, τ_a is likely to have been only slightly less than τ_b in our preparations. As explicitly stated in Eq. (17A) of our manuscript, the time-dependent term of ψ^{mc} is proportional to

$$\left[\frac{1}{1 + (C_a/C_b)} - \frac{1}{1 + (R_b/R_a)}\right],$$

which can be rearranged to

$$\left[\frac{\tau_b - \tau_a}{(C_a + C_b)(R_a + R_b)}\right].$$

(R and C symbolize resistance and capacitance, respectively, while b and a refer to the basolateral and apical membranes, respectively.) Thus, the magnitude of the time dependence would be small (as observed) if the values of τ_b and τ_a were close to one another.

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Reply to: Time-Dependent Phenomena in Voltage-Clamped Epithelia

We agree with Drs. Garcia-Diaz and Essig that in our paper (Schultz et al., 1984*a*) we did not *prove* that the time-dependent changes in the properties of the basolateral membrance of *Necturus* urinary bladder are not *entirely* due to capacitative charging.

However, further insight into this important matter can be obtained if we assume that these changes *are, in fact, entirely capacitative*. If so, the time-dependence of the voltage-divider ratio is given by

$$f(t) = f_\infty(1 - e^{-t/\tau}) + f(0)e^{-t/\tau} \quad (1)$$

where $f_\infty = r^m/(r^m + r^s)$ and $f(0) = C^s/(C^s + C^m)$ (Schultz et al., 1984*b*). Because $f(t)$ was determined both at 20 and at 90 msec, Eq. (1) can be rearranged to yield

$$\tau = \frac{20}{\ln\left\{\frac{f(0) - f_\infty}{f(20) - f_\infty}\right\}} = \frac{90}{\ln\left\{\frac{f(0) - f_\infty}{f(90) - f_\infty}\right\}}. \quad (2)$$

Now, as pointed out by Drs. Garcia-Diaz and Essig, it is quite likely that $C^s \gg C^m$ so that $f(0) \cong 1$. Morphometric studies of *Necturus* urinary bladder (Schultz et al., 1984*a*; Karnaky et al., 1984) are consistent with $f(0) \approx 0.97$.

The values of $f(20)$ and $f(90)$ when $(Na)_m$ = 3.8, 11.4 and 34.2 mM are given in the Table. Substituting these values into Eq. (2), and assuming that $f(0) = 0.97$, successive approximations yield the "best values" for f_∞ and τ given in the Table.

Thus, by 90 msec time-dependent effects due entirely to electrical capacitance should be largely (85%) dissipated. In particular, when $(Na)_m$ = 34.2 mM capacitative effects should be almost entirely (96%) completed by 90 msec, yet, the observed E^s at that time averages only 27 mV (Schultz et al., 1984*a*; Fig. 6, right), a value that is much lower than any reasonable estimate of E^s_K or the value of ψ^{cs} (73 mV) observed in the presence of amiloride.

Further, using the values of f_∞ given in the Table and the values of ${}_0g^m$ reported by Thomas et al. (1983; Table 1), which are time-independent (Schultz et al., 1984*a*), we obtain the values of ${}_0g^s$ given in the Table.

In short, even if we assume that the time-dependent changes in the values of g^s and E^s reported by Schultz et al. (1984*a*) *are entirely due to capacitative effects*, our two principal conclusions regarding the properties of the basolateral membrane of this epithelium, namely, that (i) the basolateral membrane possesses a significant conductance to an ion(s) other than K; and (ii) the conductance of that barrier increases with increasing pump rate, appear to be valid.

Finally, although we find the results of this analysis reassuring we do not wish, in any way, to minimize the importance of this hopefully constructive as well as instructive exchange. The fact is that, heretofore, little or no justification has been provided for the "timing" of electrophysiological responses of epithelia to perturbations resulting from the passage of a current pulse across the tissue (either in the voltage-clamp or current-clamp modes), particularly with regard to the estimation of the transepithelial resistance or f. As pointed out by Nagel and Essig (1982), Schultz et al. (1984*a*) and DeLong and Civian (1984), the solution of this conundrum will not be an easy matter. Ideally, this would require accurate information regarding the resistances and capacitances of the two (or more) limiting membranes encountered by transcellular current flow. Unfortunately, these values cannot be readily obtained! AC analyses directed toward this end rely

Table.

$(Na)_m$	I_{sc}[a]	$f(20)$[a]	$f(90)$	f_∞	τ	${}_0g^s$
3.8	10.3 ± 1.1	0.91 ± 0.01	0.82 ± 0.03	0.79	49	0.75
11.4	17.2 ± 2.0	0.87 ± 0.02	0.74 ± 0.04	0.71	41	1.0
34.2	25.0 ± 2.5	0.84 ± 0.04	0.73 ± 0.03	0.72	28	1.7

$(Na)_m$ is in mM; I_{sc} is in $\mu A/cm^2$; τ is in msec; and ${}_0g^s$ is in mS/cm^2. All notations are defined in Schultz et al. (1984*a*).

[a] Data previously reported by Thomas et al. (1983; Table I).

upon "curve-fitting" of experimental data to the predictions of equivalent electrical circuit models that may be oversimplifications of the complex geometries and heterogeneities of the cells that comprise even those epithelia lined by a "single layer" of cells.Thus, at the very least, DC (voltage-clamp) studies should be designed to obtain $f(t)$ as close as possible to $f(0)$, thereby providing a measure of $[C^s/(C^s + C^m)]$, and at a number of time points thereafter in the msec range. This approach could provide a means of distinguishing between time-dependent changes due to electrical capacitance and those arising from other causes.

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