J. Membrane Biol. 41, 93–100 (1978)

On the Use of Amphotericin B as a Probe to Determine Cell Membrane Resistances in Gallbladder Epithelium

Received 3 January 1978

Hénin and coworkers [1] have recently reported experiments in which the electrical resistances of the cell membranes and the paracellular pathway were calculated, in gallbladder epithelia of several species, without performing cable analysis. Their method is based on the assumption that the polyene antibiotic amphotericin B alters selectively the conductance and equivalent electromotive force of the luminal membrane of the cells, without exerting effects on these parameters at either the contralateral cell membrane or the shunt pathway. They calculated the resistances from the amphotericin B-induced changes of transepithelial potential, luminal membrane potential, ratio of cell membrane resistances, and transepithelial resistance.

The electrophysiological analysis of Hénin *et al.* can be criticized on the following grounds: (1) Their circuit analysis equations are incorrect. (2) Analysis of their data from the resistance values alone yields results that are internally inconsistent. (3) The extremely high accuracy required for these measurements in leaky epithelia makes the application of this method questionable.

1. Circuit Analysis Equations

From the analysis of the equivalent electrical circuit of the epithelium, assuming a Thevenin equivalent at each cell membrane and at the paracellular pathway (Fig. 1, Ref. 1), V_m (the mucosal membrane potential) and V_{ms} (the transepithelial potential) are given by

$$V_m = \frac{E_m(R_s + R_{sh}) + (E_s - E_{sh})R_m}{R_m + R_s + R_{sh}} \tag{1}$$

$$V_{ms} = \frac{(E_m - E_s)R_{sh} - E_{sh}(R_m + R_s)}{R_m + R_s + R_{sh}} \tag{2}$$

where E's are equivalent electromotive forces (emf's) and R's are equivalent resistances; the subscripts m, s, and sh refer to the mucosal and serosal membranes of the cells, and the paracellular pathway, respectively.

Upon exposure to amphotericin B, we assume that only R_m and E_m change, to R'_m and E'_m, respectively. The changes of V_m and V_{ms} (ΔV_m and ΔV_{ms}, respectively) are described by

$$\Delta V_m = \left(\frac{E_m}{R_t} - \frac{E'_m}{R'_t}\right)(R_s + R_{sh}) + (E_s - E_{sh})\left(\frac{R_m}{R_t} - \frac{R'_m}{R'_t}\right) \tag{3}$$

$$\Delta V_{ms} = \left(\frac{E_m - E_s}{R_t} - \frac{E'_m - E_s}{R'_t}\right) R_{sh} - E_{sh}\left(\frac{R_m + R_s}{R_t} - \frac{R'_m + R_s}{R'_t}\right) \tag{4}$$

where $R_t = R_m + R_s + R_{sh}$, and $R'_t = R'_m + R_s + R_{sh}$.

The analogous equations derived by Hénin *et al.* are:

$$\Delta V_m = \Delta E_m \frac{R_s + R_{sh}}{R'_t} \tag{5}$$

$$\Delta V_{ms} = \Delta E_m \frac{R_{sh}}{R'_t} \tag{6}$$

(the use of the expression R'_{sh} is unclear, since they assume that R_{sh} is not changed by the drug).

A comparison of Eqs. (3) and (5) indicates that Hénin *et al.* have implicitly assumed that $R_t - R'_t = 0$ and that $(R_m/R_t) - R'_m/R'_t) = 0$.

Both simplifications are wrong. From their own data, which probably includes large underestimations of R_m and R_s, $(R_t - R'_t) = 95.4$, and $(R_m/R_t) - (R'_m/R'_t) = 0.21$. The effect of the unjustified assumptions on the calculation of the resistance is large, but difficult to evaluate. As an illustration, I have calculated ΔV_m from their values of resistances and the assumptions $E_m = E_s = 70$ mV, $E_{sh} = 2$ mV, and $E'_m = 9$ mV (from the value of ΔE_m computed by Hénin *et al.*). The predicted value of ΔV_m is 31 mV (equal to the measured value) if Eq. (5) is used, and 43.4 mV if Eq. (3) is used. Most of the difference is accounted for by the second term on the right hand side of Eq. (3). Hénin *et al.* have failed to consider that, within their assumptions, changes of R_m and E_m result in changes of V_m by two mechanisms: (i) the change of E_m (taken into account in their analysis), and (ii) the change of the voltage drop caused by the other emf's of the circuit (E_s and E_{sh}) across R_m; this second mechanism was neglected.

V_{ms} can be analyzed in a similar fashion, i.e., by comparing Eqs. (4) and (6). The absolute errors are smaller, because of the large influence of E_{sh} (as compared to those of E_m and E_s on V_{ms}). It should be mentioned, however, that the assumption of a constant value of E_{sh} becomes critical, since the effect on V_{ms} of ΔE_{sh} is at least ten times larger than the effect of an identical ΔE_m.

2. *Analysis of the Data from Values of Resistances*

As shown in toad urinary bladder by Reuss and Finn [4, 5] and in rabbit urinary bladder by Lewis, Eaton and Diamond (3), R_m, R_s and R_{sh} can be calculated from R_{ep} (transepithelial resistance) and $a (= R_m/R_s)$ before and after a selective change of R_m. This was achieved by ionic replacements or by the use of amiloride.

The appropriate equations are:

$$R_{sh} = \frac{R_t R'_t (a' - a)}{R_t(a' + 1) - R'_t(a + 1)} \tag{7}$$

$$[R_s(a + 1)]^{-1} = R_t^{-1} - R_{sh}^{-1} \tag{8}$$

$$\frac{R_m}{R_s} = a. \tag{9}$$

Where R_t and a represent values before and R'_t and a' indicate values after the addition of the drug.

Table 1. Comparison of resistance values (in Ωcm^2) obtained from the analysis of Hénin *et al.* (*A*), and from the use of Eqs. (7)–(10) (*B*)

Species	R_m	R_s	R_{sh}	
Rabbit	156	143	20.8	A
	29	27	28.2	B
Guinea pig	280	295	69.7	A
	−195[a]	−206[a]	53.5	B
Goose[b]	625	378	29.9	A
	[b]	[b]	29.0	B
Tortoise	1607	861	99.1	A
	361	212	104.6	B
Trout	528	212	29.4	A
	95	36	30.7	B

[a] Negative values of R_m and R_s are obtained because $R_t' > R_t$, whereas $a' < a$.
[b] $R_m + R_s = \infty$, because $R_t' = R_t$, whereas $a' < a$.

Eqs. (7)–(9) can be applied to the results communicated by Hénin *et al.* Since the same assumption is made (i.e., that only R_m changes), the results should be identical to those obtained from their calculation. The comparison is shown in Table 1. It can be seen that the cell membrane resistance results differ by a factor of at least 4.

These discrepancies are the consequence of the approximations involved by the use of Eqs. (5) and (6). Recent experiments in *Necturus* gallbladder (2) showed that shortly after the action of amphotericin B the K/Na selectivity of the shunt pathway increases by about 50%. Assuming that all other transference numbers remain constant, the increase of t_K could account for a change of R_{sh} of 1.5 to 2%. This change is large enough to produce an underestimation of R_m and R_s by a factor of 2. Furthermore, even shortly after the addition of amphotericin B, R_s decreases, as shown by cable analysis experiments (2). This change can also result in underestimation of R_m and R_s.

3. *Accuracy Required for the Analysis*

In tight epithelia, amiloride causes large changes in both a and R_{ep}. In leaky epithelia, large changes of R_m result in small changes of R_{ep}, because of the low value of R_{sh}. From mean values of R_{ep} and a in *Necturus* gallbladder, before and after amphotericin B, it can be estimated that an error of R_{ep}' of only $1\,\Omega cm^2$ (0.3%) results in errors of 10–17% in the values of R_m and R_s. If the resistances of the cell membranes in rabbit gallbladder are similar to those of *Necturus*, the situation is even worse, because of the lower R_{sh}. If, for instance, $(R_a + R_b) = 5{,}000\,\Omega cm^2$, and $R_{sh} = 20\,\Omega cm^2$, a change of a from 1.5 (control) to 0.4 (amphotericin B) should result, if R_s and R_{sh} remain constant, in a change of R_{ep} of $0.062\,\Omega cm^2$. If ΔR_{ep} is overestimated by $0.01\,\Omega cm^2$, R_m is overestimated by almost $1{,}000\,\Omega cm^2$, and if ΔR_t is underestimated by the same amount, R_m is underestimated by about $400\,\Omega cm^2$. It is questionable if R_{ep} can be measured with an

accuracy of $0.003\,\Omega\,cm^2$, needed to reduce the error in the estimation of R_m and R_s to about 10%.

Luis Reuss
Department of Physiology and Biophysics
Washington University School of Medicine
660 South Euclid Avenue
St. Louis, MO 63110

References

1. Hénin, S., Cremaschi, D., Schettino, T., Meyer, G., Donin, C.L.L., Cotelli, F. 1977. Electrical parameters in gallbladders of different species. Their contribution to the origin of the transmural potential difference. *J. Membrane Biol.* **34**:73
2. Reuss, L. Effects of amphotericin B on the electrical properties of *Necturus* gallbladder: Intracellular microelectrode studies. *J. Membrane Biol.* (*in press*)
3. Lewis, S.A., Eaton, D.C., Diamond, J.M. 1976. The mechanism of Na^+ transport by rabbit urinary bladder. *J. Membrane Biol.* **28**:41
4. Reuss, L., Finn, A.L. 1974. Passive electrical properties of toad urinary bladder epithelium. Intercellular coupling and transepithelial and shunt conductances. *J. Gen. Physiol.* **64**:1
5. Reuss, L., Finn, A.L. 1975. Effects of changes in the composition of the mucosal solution on the electrical properties of the toad urinary bladder epithelium. *J. Membrane Biol.* **20**:191

Reply to: On the Use of Amphotericin B as a Probe to Determine Cell Membrane Resistances in Gallbladder Epithelium

Received 14 March 1978

In his letter Dr. Reuss criticizes the method used by us [5] to measure membrane and shunt resistances in a leaky epithelium such as gallbladder.

We agree with him that the flat cable analysis is, in principle, more convenient and reliable. On the other hand, the study of epithelia should not be limited to those substrates which present the exact geometrical characteristics necessary to evaluate the distance between the current injecting and the recording electrodes (i.e., no foldings), which is the crucial point for cable analysis.

For this reason we suggested the method based on Amphotericin B action. As a matter of fact, at least for tight epithelia, the use of a selective drug able to change only a few electrical parameters (e.g., amiloride and nystatin) is now widely accepted by many authors, including Reuss himself [3, 6, 7, 8]. Thus, the only point at issue is whether this method can be applied to leaky epithelia.

On the basis of what we will discuss in this letter, it should become clear that:

1) The method we suggested is essentially founded on variations of transepithelial and intracellular potentials, rather than on changes of transepithelial resistance. The former can be easily measured, whereas changes in the latter are too slight in a leaky epithelium to be measured with sufficient precision.

2) Our simplified equations are not derived from those approximations assumed by Reuss. We considered that the overall emf of the cell membranes and the shunt was nearly equal to zero, or that the shunt resistance was not affected by amphotericin B.

3) Our assumptions are not hypothetical, but based on experimental evidences.

Validity of the Equations used in the Circuit Analysis

The first of Reuss's criticisms concerns the approximations introduced in the mathematical analysis of the circuit. Our equations are simplifications of the general equations (1) and (2) in Reuss's letter.

On the basis of Eqs. (1) and (2), changes in V_m and V_{ms} are:

$$\Delta V_m = E_m\left(\frac{R_s+R_{sh}}{R_t}\right) - E'_m\left(\frac{R_s+R'_{sh}}{R'_t}\right) + (E_s-E_{sh})\left(\frac{R_m}{R_t}-\frac{R'_m}{R'_t}\right) \tag{a}$$

$$\Delta V_{ms} = (E_m-E_s)\frac{R_{sh}}{R_t} - (E'_m-E_s)\frac{R'_{sh}}{R'_t} - E_{sh}\left(\frac{R_m+R_s}{R_t}-\frac{R'_m+R_s}{R'_t}\right). \tag{b}$$

The symbols used are the same as employed by Reuss. Equations (*a*) and (*b*) can be derived from Equations (1) and (2) since E_{sh} and R_s are not affected by the polyene (*see* ref. 1). Equations (*a*) and (*b*) do, however, contain variables which cannot be measured and they cannot therefore be used in this form. They can be reduced to a simple and useful form if, under control conditions, $E_m-E_s+E_{sh}=0$, so that no current circulates through $R_m+R_s+R_{sh}$. In this case Equation (*a*) becomes:

$$\Delta V_m = \Delta E_m\left(\frac{R_s+R'_{sh}}{R'_t}\right) \tag{c}$$

which is identical to Eq. (1) of our previous paper (ref. 5) and Eq. (*b*) in the same way becomes:

$$\Delta V_{ms} = \Delta E_m\left(\frac{R'_{sh}}{R'_t}\right). \tag{d}$$

This is identical to Eq. (2) of [5]. In fact, the current circulating through $R_m+R_s+R_{sh}$ after treatment with amphotericin B is:

$$i' = \frac{E'_m-E_s+E_{sh}}{R'_t} \tag{e}$$

and this can be divided into two components:

$$i' = \frac{E_m-E_s+E_{sh}}{R'_t} - \frac{\Delta E_m}{R'_t}. \tag{f}$$

The first term on the right hand side is cancelled if $E_m-E_s+E_{sh}=0$. The current under this particular condition is generated only by ΔE_m. This situation can be that of rabbit gallbladder [4] where $E_{sh} \ll E_s$ and $E_m \simeq E_s$.

A second possibility to obtain simplified equations consists of assuming $R_{sh}=R'_{sh}$. As a matter of fact R_{sh} nearly always remained constant for at least 10 min after the addition of amphotericin B. There were rare occasions when R_{sh} did change and in these circumstances, as stated on page 76 and 80 of our original paper, we rejected the experiment. When R_{sh} remained constant, Eq. (3) of our paper and Eqs. (3) and (4) of Reuss's letter are equally valid. Reuss's Eqs. (5) and (6) may be derived only if $R_t - R'_t = 0$ and $(R_m/R_t)-(R'_m/R'_t)=0$.

We completely agree with Reuss that in our data $R_t \neq R'_t$ and $(R_m/R_t)\neq(R'_m/R'_t)$. We too have emphasized that R_m is largely decreased by the polyene [1, 5]. This point is, however, entirely irrelevant, since *we never used these wrong assumptions, either explicitly or implicitly in our original analysis of the data.*

In our calculations Reuss's Eqs. (3) and (4) are only simplified further when considering a ratio [*see* our Eq. (3)]. As a matter of fact, the ratio $\Delta V_m/\Delta V_{ms}$ is the crucial datum used by us. On the basis of Reuss's Eqs. (3) and (4) we obtain:

$$\frac{\Delta V_m}{\Delta V_{ms}}=\frac{R_s+R_{sh}}{R_{sh}}=\frac{R_s}{R_{sh}}+1 \tag{g}$$

which is identical to Eq. (3) of our paper (as $R_{sh}=R'_{sh}$) (*see also* ref. 3). From it the equations used in the calculations are directly derived (Eqs. (4), (8), (10) of ref. 1).

As a conclusion, we can derive the practical equations used in our paper, either considering $E_m - E_s + E_{sh}=0$, which is valid at least in rabbit gallbladder, or considering $R_{sh}=R'_{sh}$, which is valid for all the tested species.

Evaluation of the Errors Inherent in the Method

First of all Reuss *wrongly* supposes that we assume $R_t=R'_t$ and $(R_m/R_t)=(R'_m/R'_t)$. On this basis he states that "the effect of the unjustified assumptions on the calculation of the resistances is large." As an illustration he computes ΔV_m from our values of resistances and on the assumption that $E_m=E_s=70$ mV, $E_{sh}=2$ mV, and $E'_m=9$ mV. Calculations were performed using, he says, his Eqs. (3) and (5) to evaluate the effect of the simplification. He points out that the two results are very different, i.e., 43.4 mV with Eq. (3) and 31 mV with Eq. (5). We have repeated his calculations and we have found $\Delta V_m = 43.7$ mV with Eq. (3) and 44.1 mV with Eq. (5):

$$\Delta V_m=\left(\frac{70}{320}-\frac{9}{227}\right)(143+21)+(70-2)\left(\frac{156}{320}-\frac{63}{227}\right)=43.7 \tag{3}$$

$$\Delta V_m=(70-9)\left(\frac{143+21}{227}\right)=44.1. \tag{5}$$

From the reported calculations it is clear that R_t (320) $\neq R'_t$ (227) and that $(R_m/R_t)(156/320)\neq(R'_m/R'_t)(63/227)$. Nevertheless, the difference between the two values of ΔV_m of only 0.9% shows that the two equations give virtually the same result when $E_m - E_s + E_{sh} \simeq 0$, as assumed in Reuss's example.

Furthermore it must be pointed out that the second term on the right hand side of Eq. (3) is not negligible and that Eq. (5) *is not equal* to the first term on the right hand side of Eq. (3), so that the statement that "most of the difference is accounted for by the second term" is incorrect. The two values 31 and 43.4 mV, computed by Reuss, are easily obtained if we wrongly use the first term of Eq. (3) instead of Eq. (5) in the calculations.

Moreover, it is noteworthy that, using $E_m = E_s = 60\,\text{mV}$, $E_{sh} = 2\,\text{mV}$ and $E'_m = 16\,\text{mV}$, values which are more consistent with our data (*see* refs. 4 and 5), we obtain $\Delta V_m = 31.2\,\text{mV}$ with Eq. (3) and $\Delta V_m = 31.8\,\text{mV}$ with Eq. (5) *vs.* our experimental result $\Delta V_m = 31.3\,\text{mV}$.

Another main criticism by Reuss is pointed out in Table 1 of his letter. This table compares R_m, R_s and R_{sh}, computed by us, with the same parameters calculated with Reuss's Eqs. (7), (8) and (9) using our experimental results for R_{ep}, R'_{ep} and α'.[1]

Not only are the series of data largely different, but in the second series some values are merely absurd. In Reuss's opinion these discrepancies are again due to the "approximation involved in the use of Eqs. (5) and (6)". On the contrary, the large error is clearly due to the use of Eqs. (7), (8) and (9). They are based on R_{ep} variations and so they are useful *only* in tight epithelia where the drug causes large relative changes in R_{ep}. They cannot be easily applied in leaky epithelia where absolute and relative R_{ep} variations are necessarily very small. Their use in leaky epithelia requires a very high accuracy in R_{ep} measurement, an accuracy which is technically impossible to reach. For this reason the use of our experimentally measured R_{ep} and R'_{ep} values to calculate R_m, R_s and R_{sh} gives inconsistent results, using Eqs. (7), (8) and (9). The inherent inaccuracy of the method is clearly amplified if the calculations are performed, as Reuss does, on mean values instead of on paired data taken from the same experiment.

The use of Eqs. (7), (8) and (9) is to be avoided for a second reason. They are derived [as well as Eqs. (3) and (4)] on the assumptions that $R_{sh} = R'_{sh}$, but the mathematical role of this parameter is such that small R_{sh} variations, caused by the antibiotic, generate large errors in the calculated R_m and R_s.

In leaky epithelia the method based on the change in potentials (ΔV_m and ΔV_{ms}), which is large and can be accurately determined, must be preferred. Even if the absolute ΔV_{ms} value is of few mV it is still accurately measurable since $\Delta V_{ms}/V_{ms} \geqq 100\,\%$. It is noteworthy that even with this method R_{ep} is involved in the calculation of R_m and R_s, but its position in our equations [5] is such that an error of, say, 10% in R_{ep} measurement causes an error of 10% in R_{sh}, R_m and R_s. Also errors due to R_{sh} alterations caused by the antibiotic are minimized even when we employ equations in which R_{sh} is assumed to be equal to R'_{sh}.

For instance a $\pm 10\,\%$ alteration of R_{sh} causes an error of $\pm 7.3\,\%$ in the calculated R_m and R_s.

As a conclusion, for a leaky epithelium, the method based on resistance change and the method based on voltage variations require very different degrees of accuracy. They are not equivalent, even if both are derived on the same theoretical assumptions.

Validity of the Assumptions

Concerning the assumptions $E_m - E_s + E_{sh} \simeq 0$ and $R_{sh} \simeq R'_{sh}$, we have already emphasized that the former is valid at least for rabbit gallbladder [4], the latter is generally valid during the 10 min after treatment [1, 5]. Evidence concerning E_{sh}, E_s and R_s constancy during the same period are reported in ref. 1.

We wish to stress that on pages 80–81 of [5] we stated that it was impossible to perform the analysis on toad gallbladder because the above conditions could not be maintained for the time needed to carry out the necessary measurements. Similar difficulties are likely to arise in other amphibian gallbladders such as *Necturus*, which is used by Reuss.

---

1 In Reuss's Eqs. (7), (8) and (9), the transepithelial resistance is reported as R_t instead of R_{ep}.

We think, therefore, that our assumptions are sound. The results obtained for eterotherms, in very good agreement with those obtained with the flat cable analysis by Frömter [2] in *Necturus*, corroborate this conclusion.

Silvio Hénin and Dario Cremaschi
Istituto di Fisiologia Generale e di Chimica
Biologica Università di Milano,
via Mangiagalli 32, Milano (Italia)
Istituto di Fisiologia Generale,
Università di Bari, via Amendola 165/A,
Bari (Italia)

References

1. Cremaschi, D., Hénin, S., Meyer, G., Bacciola, T. 1977. Does Amphotericin B unmask an electrogenic Na^+ pump in rabbit gallbladder? Shift of gallbladders with negative to gallbladders with positive transepithelial p.d.'s. *J. Membrane Biol.* **34**:55
2. Frömter, E. 1972. The route of passive ion movement through the epithelium of *Necturus* gallbladder. *J. Membrane Biol.* **8**:259
3. Frömter, E., Gebler, B. 1977. Electrical properties of Amphibian urinary bladder epithelia. III. The cell membrane resistance and the effect of amiloride. *Pfluegers Arch.* **371**:99
4. Hénin, S., Cremaschi, D. 1975. Transcellular ion route in rabbit gallbladder. Electrical properties of epithelial cells. *Pfluegers Arch.* **355**:125
5. Hénin, S., Cremaschi, D., Schettino, T., Meyer, G., Donin, C.L.L., Cotelli, F. 1977. Electrical parameters in gallbladders of different species. Their contribution to the origin of the transmural potential difference. *J. Membrane Biol.* **34**:73
6. Lewis, S.A., Eaton, D.C., Clausen, C., Diamond, J.M. 1977. Nystatin as a probe for investigating the electrical properties of a tight epithelium. *J. Gen. Physiol.* **70**:427
7. Lewis, S.A., Eaton, D.C., Diamond, J.M. 1976. The mechanism of Na^+ transport by rabbit urinary bladder. *J. Membrane Biol.* **28**:41
8. Reuss, L., Finn, A.L. 1975. Effects of changes in the composition of the mucosal solution on the electrical properties of the toad urinary bladder epithelium. *J. Membrane Biol.* **20**:191