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On the Effects of Amphotericin B and Ouabain on the Electrical Potentials of *Necturus* Gallbladder

Recently two papers have appeared in *The Journal of Membrane Biology* which address themselves to the question whether the Na-K-ATPase (the pump) in the *Necturus* gallbladder epithelium is electrogenic (rheogenic) or neutral. The two researchers were using the same methods – microelectrodes and amphotericin B and the same model of the tissue. Despite these similarities, they reach exactly the opposite conclusions: Graf and Giebisch (1979) argue that the pump unmasked by amphotericin B is electrogenic, whereas Reuss (1978) argues that the results are consistent with a neutral pump. Other reports are also in conflict: van Os and Slegers (1975) and Reuss, Bello-Reuss and Grady (1979), using ouabain and electrodes, claim that the pump is neutral. Rose and Nahrwold (1976), however, find that the pump is electrogenic.

The purpose of this letter is (i) to show that these discrepancies are due to the use of a too simple model of the tissue; (ii) to develop a more useful model based upon ionic currents, mass balance, and electroneutrality in order to describe which depolarizations can be expected from the action of ouabain.

Effects of AmphotericinB

Any effects of a electrogenic Na^+-transport should be enhanced if the Na^+-transport was stimulated. Amphotericin B increases the Na^+ permeability of the mucosal membrane of the gallbladder (Cremaschi et al., 1977; Rose & Nahrwold, 1976; Reuss, 1978; Graf & Giebisch, 1979), the mucosal membrane ceases to be the rate-limiting step for Na^+ transport, intracellular Na^+ increases, and the ATPase-mediated Na^+ transport is increased.

The two reports using amphotericin B and microelectrodes both analyze their results by means of a model of the cell based upon one resistor in series with one battery for each membrane and for the paracellular shunt. The electromotive force at the serosal membrane E_{23} is taken as a measure of the electrogenicity of the Na^+ pump. E_{23} is approximated by

$$E_{23} \cong V_{23} - V_{13}\frac{R_{23}}{R_{13}} \tag{1a}$$

where V_{23} is the voltage measured across the serosal membrane and V_{13} the voltage across the epithelium, R_{13} is the resistance of the tissue (*see*, e.g., Fig. 1 of Graf & Giebisch, 1979).

The main difference between the two reports appears to be their assessment of the value of the resistance of the serosal membrane R_{23}; the subsequent subtraction of the two terms on the right-hand side of Eq. (1a) causes the different assessments in E_{23}. The first report (Graf & Giebisch, 1979) assumes that the serosal membrane resistance is constant: Amphotericin B only acts at the mucosal membrane, if the intracellular Na^+ should increase while the K^+ activity decreased (Cremaschi et al., 1977) the resistance should not decrease because the partial conductance of K^+ for the serosal membrane is larger than for Na^+. Via an estimate of the remainder of the parameters in the three-resistor model (this estimate is about the same in the two studies), Graf and Giebisch (1979) conclude that the electromotive force of the battery on the serosal membrane, E_{23}, increases from -80 to -120 mV during the application of amphotericin B, wherefore an electrogenic pump has been unmasked. Reuss et al. (1979), however, makes an estimation of R_{23} during the application of amphotericin B using cable analysis and voltage divider ratio and finds that R_{23} decreases. When these authors evaluate the electromotive force of the battery of the serosal membrane, they find that it decreases from -80 to -60 mV in direct contrast to the conclusion of Graf and Giebisch (1979).

There are several uncertainties in the arguments used. Firstly, the value and meaning of R_{23} is unclear. This resistance is determined partly from the voltages induced by a transmural current, and only part of the basolateral membrane partakes in the transcellular current-pathway; If there is a voltage gradient in the lateral intercellular spaces and the cell is isopotential during the application of a transmural current, the voltage across the serosal membrane at the apical end would be smaller than at the basal end. If, e.g., the lateral spaces had a resistance which was about 50% of the whole transepithelial resistance, as inferred by Frömter (1972) and Simon et al. (1981) and the resistance of the mucosal membrane was about twice that of the serosal membrane (e.g., Frömter 1972), transepithelially applied current would in fact pass into the cell from the lateral intercellular spaces, a situation the three resistor model does not allow. As a result, the current would pass across this membrane predominantly across the basal part of the serosal membrane, and the estimated impedance will be too high (Zeuthen, 1976). Now the $Na^+ - K^+$ ATPase is situated all over the lateral membrane (DiBona & Mills, 1979), and it is likely that the current generated by the pump is evenly distributed over the whole of the basolateral membrane (and the apical membrane as well). The resistance encountered by the current arising from the pump will therefore be entirely different from the resistance derived from the transcellular measurements.

Secondly, the electromotive force E_{23} is a sum of a diffusion potential and a current-generated potential. If the diffusion potential decreases as a result of the increased intracellular Na^+ and decreased K^+-activity (the membrane is predominantly K^+-permeable), the current-generated potential *per se* may actually increase even if the total electromotive force decreases. Thus even if R_{23} was well defined it is difficult to argue about the electrogenicity of the pump from changes in the electromotive force E_{23} (*see also* Reuss et al., 1979).

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Effects of Ouabain

The effect of ouabain on the intracellular electrical potential of *Necturus* gallbladder was slow (Reuss et al. 1979; van Os & Slegers, 1975; Zeuthen, 1978); after 30 min the depolarization was only of the order 5 to 10 mV or 10%; no abrupt depolarization was observed. From these findings it was suggested (Reuss et al., 1979, van Os & Slegers, 1975) that the Na^+-pump in *Necturus* gallbladder was neutral: As the secretion in isotonic with the luminal solution, the net transmural transport of Na^+ could be assessed as a function of time. If this Na^+-flux results from a $Na^+ - K^+$ ATPase with a coupling ratio of 3:2, the current flowing through the cell could be calculated as 12.7 $\mu A\,cm^{-2}$. The voltage across the basolateral membrane (2.3 $k\Omega cm^2$, Reuss et al., 1979) caused by this current would amount to 29 mV and was taken to present the contribution of the electrogenic pump to the intracellular potential. However, no such depolarization was observed, and consequently the pump was suspected to be neutral.

One reason why the simple three-resistor model predicts a ouabain-induced depolarization of 29 mV (instead of the thermodynamically possible 10.4 mV, *see* below) is because the value of the resistance of the basolateral membrane determined from electrical measurements may be larger than the actual value. As argued above, the $Na^+ - K^+$ ATPase is evenly distributed over the serosal membrane and its current can cross the whole membrane; the current applied for measuring the resistance of the serosal membrane crosses only part of the serosal membrane. Thus if the resistance of the basolateral membrane was overestimated by a factor of 3, the expected depolarization induced by ouabain could still be within the thermodynamically possible 10.4 mV even if predicted by the simple electrical model. The slow response of ouabain could be explained by the diffusive delay through the connective tissue.

A Model and the Effects of Ouabain

The compartment from which Na and Cl is absorbed is called compartment 1. This equals the lumen of the gallbladder. Compartment 2 is the cell interior, and the compartment into which Na and Cl is secreted (blood) is compartment 3.

Mass balance gives:

$$T_{Na}^{12} + \phi_{Na}^{12} + \phi_{Na}^{23} + p_{Na}^{23} = h\frac{dNa_2^+}{dt} \tag{1}$$

$$\phi_K^{12} + \phi_K^{23} + p_K^{23} = h\frac{dK_2^+}{dt} \tag{2}$$

$$T_{Cl}^{12} + \phi_{Cl}^{12} + \phi_{Cl}^{23} = h\frac{dCl_2^-}{dt}. \tag{3}$$

$T_{Na}^{12} = T_{Cl}^{12}$ describes the neutral influx of Na^+ (from *1* to *2*) coupled to T_{Cl}^{12}, the neutral flux of Cl^-. p_{Na}^{23} is the active (via a $Na^+ - K^+$ activated ATPase) efflux of Na^+ coupled to the active influx of K^+ (p_K^{23}) via the coupling-ratio, r, defined by

$$-p_{Na}^{23} = r p_K^{23} \tag{4}$$

ϕ are the passive fluxes governed by the constant-field equation, i.e:

$$\phi_{Na}^{12} = h P_{Na}^{12} \cdot \frac{\psi_{12} \cdot F}{RT} \cdot \frac{Na_1^+ - Na_2^+ \exp\frac{[\psi_{12} \cdot F]}{RT}}{1 - \exp\left[\frac{\psi_{12} \cdot F}{RT}\right]} \tag{5}$$

where P_{Na}^{12} is the passive permeability of the membrane separating compartments *1* and *2*, which have the potential difference $\psi_{12} = \psi_2 - \psi_1$. In a leaky epithelium bathed in identical solutions on both sides $\psi_{12} = -\psi_{23}$ which simplifies Eqs. (1), (2), and (3) very much. h is the cell volume per unit area of epithelia, thus the terms on the right-hand side describe the rate of change in the amounts of intracellular ions.

In a steady state the right-hand sides of Eqs. (1), (2), and (3) are zero. Multiplying Eq. (2) with r, adding Eqs. (1) and (3), and inserting Eq. (5) (using $\psi_{12} = -\psi_{23}$ for a leaky epithelium) gives, by simple algebraic manipulations,

$$\psi_{12} = -\psi_{23} = \frac{RT}{F} \cdot \ln \frac{r P_K K_1^+ + P_{Na} Na_1^+ + P_{Cl} Cl_2^-}{r P_K K_2^+ + P_{Na} Na_2^+ + P_{Cl} Cl_1^-}. \tag{6}$$

$P_K = P_K^{12} + P_K^{23}$ is the *total* K^+ permeability of the cell; similarly for P_{Na} and P_{Cl}.

The model will predict the depolarization caused by ouabain added to the solution in compartment *3*. Immediately after the application of ouabain

$$p_{Na}^{12} = p_K^{23} = 0$$

and due to the requirement of electroneutrality

$$\frac{dNa_2^+}{dt} + \frac{dK_2^+}{dt} = \frac{dCl_2^-}{dt}. \tag{7}$$

Adding the left-hand sides of Eqs. (1) and (2) and equating them to the left-hand side of Eq. (3) gives, after simple algebraic manipulation,

$$\psi_{12}^{ouab} = \frac{RT}{F} \ln \frac{P_K K_1^+ + P_{Na} Na_1^+ + P_{Cl} Cl_2^-}{P_K K_2^+ + P_{Na} Na_2^+ + P_{Cl} Cl_1^-}. \tag{8}$$

Thus expression (8) applies for a nonsteady state as long as the changes are so slow (slower than the time constant of the membrane) that the electrical field is constant within the membranes. The depolarization caused by ouabain is thus, if

$\psi_{12}^{ouab} - \psi_{12} = \Delta\psi^{ouab}$:

$$\Delta\psi^{ouab} = \frac{RT}{F} \ln \frac{P_K K_1^+ + P_{Na} Na_1^+ + P_{Cl} Cl_2^-}{P_K K_2^+ + P_{Na} Na_2^+ + P_{Cl} Cl_1^-} \cdot \frac{r P_K K_2^+ + P_{Na} Na_2^+ + P_{Cl} Cl_1^-}{r P_K K_1^+ + P_{Na} Na_1^+ + P_{Cl} Cl_2^-}. \tag{9}$$

Two cases will be distinguished:

a) If P_K is so large that $P_K K_1^+ \gg P_{Na} Na_1^+ + P_{Cl} Cl_2^-$, i.e., $P_K \gg 50\, P_{Na}$, then the intracellular potential ψ_1 will be given by the equilibrium potential of K^+ whether ouabain is added (Eq. (8)) or not (Eq. (9)); $\Delta\psi^{ouab}$ will be close to zero whether the pump is electrogenic $r = 1$ or not $r > 1$.

b) If $P_K K_2^+ \gg P_{Na} Na_2^+ + P_{Cl} Cl_1^-$, i.e., $P_K \gg 0.25\, P_{Na}$, then (9) reduces to

$$\Delta\psi^{ouab} = \frac{RT}{F} \ln r + \frac{RT}{F} \ln \frac{P_K K_1^+ + P_{Na} Na_1^+ + P_{Cl} Cl_2^-}{r P_K K_1^+ + P_{Na} Na_1^+ + P_{Cl} Cl_2^-} \tag{10}$$

as the last term is always negative the maximal depolarization $\Delta\psi^{ouab,\,max}$ is given by the term $RT/F \cdot \ln r$ which is positive. Thus

$$\Delta\psi_{r=1}^{ouab,\,max} = 0\ mV \tag{11}$$

$$\Delta\psi_{r=1.5}^{ouab,\,max} = 10.4\ mV \tag{12}$$

$$\Delta\psi_{r=5}^{ouab,\,max} = 28.1\ mV. \tag{13}$$

A model based only on steady states has been presented elsewhere (Thomas, 1972).

In the choroid plexus ouabain caused an abrupt depolarization of 10.2 mV (Zeuthen & Wright, 1978, 1981; Zeuthen, 1981). In the rat kidney proximal tubule Frömter and Gessner (1975) recorded an abrupt depolarization of 10.6 mV. In these two studies the pump was poisoned momentarily. It is strong evidence in favor of the model that these two values are so close to the value predicted: 10.4 mV for a coupling ratio of $r=1.5$. In *Necturus* gallbladder ouabain acts gradually over a period of 30 min due to the diffusional delay in the connective tissue and no abrupt depolarization is observed. Thus the experimental evidence does not allow a firm choice between the following three cases: (i) The pump is neutral $r=1$. (ii) The pump is electrogenic, but P_K is so large that the intracellular electrical potential always equals the equilibrium potential for K^+. (iii) The pump is electrogenic with $r=1.5$; if in this case $P_K=17\,P_{Na}$ (Eq. (12)) the depolarization would in fact be 6 mV as observed by Reuss et al., 1979.

Conclusion

The experimental evidence against an electrogenic pump in the *Necturus* gallbladder is nonexistent because they are based upon insufficient knowledge about the true resistance of the serosal membrane: The data available are not incompatible with the existence of an electrogenic pump with a coupling ratio of 3:2 – if an ionic model of the cell behavior is used.

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Reply to: On the Effects of Amphotericin B and Ouabain on the Electrical Potentials of *Necturus* Gallbladder

In our paper "Intracellular sodium activity and sodium transport in *Necturus* gallbladder epithelium" we had shown that luminal application of amphotericin B reduces the electric potential, the resistance, and the permselectivity (P_K/P_{Na}) of the luminal membrane, it increases intracellular sodium activity and active transepithelial sodium transport and produces a sustained serosa positive transepithelial potential. On the basis of a simple equivalent circuit and the assumption that the basolateral membrane resistance is not immediately affected by the drug, we calculated a rise of the basolateral membrane emf. This increase in the emf was attributed to a rheogenic pump since changes in the intracellular ion activities would tend to reduce rather than increase the emf. Recently Reuss (1978), using cable analysis, has calculated a decrease of the basolateral membrane resistance and consequently a decrease of its emf (for otherwise similar experimental conditions). Because of this disparity we feel that the question of whether the basolateral sodium pump is rheogenic or not needs reevaluation. Such a reevaluation must consider:

a) A more realistic electrical equivalent circuit of the epithelium, i.e., a distributed resistance along the lateral intercellular space in series with the tight junctional resistance (Clausen, Lewis & Diamond, 1979; Boulpaep & Sackin, 1980). Such a resistance will tend to attenuate an electrogenic or rheogenic component of the pump, if the pumps are uniformly distributed along the basal and lateral membranes.

b) The exact distribution of ionic pumps along such a distributed pathway (Mills & Dibona, 1980).

c) The dependence of the transepithelial and cellular resistances on the functional state of the epithelium. (Suzuki & Frömter, 1977).

d) Possible changes of the emf at the tight junctions (Curci & Frömter, 1979).

e) Alteration of passive permeability properties of the individual membranes to changes of intracellular ion activities or apparent changes of membrane permeability caused by locally elevated or depressed extracellular ion activities (Curci & Frömter, 1979).

f) The nature of NaCl entry across the luminal membrane and also of Cl^- exit across the basolateral membrane (Reuss, 1979) which seems to be electrically neutral.

Many of the above comments are in agreement with those of Zeuthen. However, Zeuthen's analysis has chosen to ignore perhaps the most significant features of epithelial transport.

First and foremost, epithelia perform vectoral transport because they possess series membranes of differing transport properties in parallel with a diffusional barrier. Consequently, treating the epithelium as a nonpolarized cell is inaccurate.

Second, in the derivation of the equation to account for the coupling ratio of the pump, the passive and neutral influx of Na^+ has been ignored and consequently the derived equation (which is similar to that used by Mullins and Noda (1963) in their studies of muscle membranes) will underestimate the true electrogenic component of the pump.

Lastly, a cell whether polarized or not is an interacting dynamic system and perhaps a more sophisticated modeling approach such as that used by Mikulecky and Thomas (1978) will be able to accurately reproduce the response of this epithelium to ionic and metabolic pertubations.

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