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APPENDIX: KINETICS OF FATTY ACID-MEDIATED PROTON MOVEMENT ACROSS SMALL UNILAMELLAR VESICLES

Background Proton Leak. When a pH gradient is imposed upon SUV prior to any addition of FA, for instance by adding KOH to the external medium (Figure 3B), we assume the proton flux across the membrane (J_H) to be equal to an unspecific (background) proton leak, J_H^b (significant only in the presence of valinomycin). At the small pH gradients applied J_H^b is proportional to the pH gradient across the SUV according to the following phenomenological relationship (Arents et al., 1981; Westerhoff & Van Dam, 1987; Kamp, 1991):

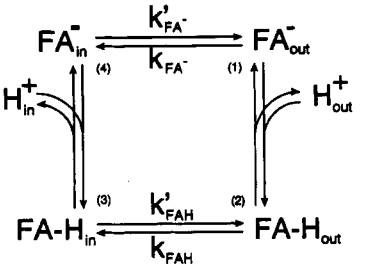
$$J_H^b = L(pH_{out} - pH_{in}) \quad (1)$$

J_H^b is related to the change in internal pH as follows:

$$J_H^b = J_H = B_{in}\, d(pH_{in})/dt \quad [nmol/(min, \mu mol\ of\ PC)] \quad (2)$$

where B_{in} is the internal buffer capacity, which can be calculated to be approximately constant over the narrow pH ranges in our experiments [B_{in} = 21 nmol of H^+/(pH unit, μmol of PC), for vesicles of 25-nm diameter containing 100 mM Hepes buffer in the entrapped aqueous volume]. Since the external pH is strongly buffered, one finds by integrating that eqs 1 and 2 predict the internal pH to increase exponentially toward pH_{out} with a relaxation rate constant k_b ($=L/B_{in}$).

Fatty Acid-Mediated Proton Leak. When a pH gradient is generated instantaneously upon the addition of an aliquot of N mol of FA (per mol of PC) to the external buffer (Figure 3B), the subsequent slow proton efflux will equal the background leak plus the rate at which protons are transferred by the FA. The latter involves transbilayer movement of both un-ionized and ionized FA. We apply the following kinetic diagram for the flip-flop and (de)protonation reactions of FA in a membrane:



(3)

We assume that the protonation and deprotonation reactions are extremely fast, so that at either side of the membrane the protonated and unprotonated forms of FA are always at equilibrium; i.e., the following buffer equations apply at all times:

$$pH_{in} = pK_a - \log[p(FAH_{in})/p(FA^-_{in})] \quad (4)$$

$$pH_{out} = pK_a - \log[p(FAH_{out})/p(FA^-_{out})] \quad (5)$$

where $p(X)$ denotes the probability of the FA to be in state X.

$p(FAH_{out})$, $p(FA^-_{out})$, $p(FAH_{in})$, and $p(FA^-_{in})$ can be expressed in terms of pH_{in} and pH_{out} by combining expressions 4 and 5 with the assumptions that (i) the sum of the probabilities of all four states is 1, (ii) at the time scale of our proton efflux experiments, the transmembrane distribution of FAH corresponds to the equilibrium distribution (i.e., $1/k_{FAH}$ and $1/k'_{FAH}$ are much shorter than the time scale of observation), and (iii) flip-flop of un-ionized FA is equally fast in both directions ($k_{FAH} = k'_{FAH}$). As a consequence

$$p(FA^-_{out}) = 1/(1 + 2 \times 10^{(pK_a - pH_{out})} + 10^{(pH_{in} - pH_{out})}) \quad (6)$$

$$p(FA^-_{in}) = 10^{(pH_{in} - pH_{out})}/(1 + 2 \times 10^{(pK_a - pH_{out})} + 10^{(pH_{in} - pH_{out})}) \quad (7)$$

$$p(FAH_{in}) = p(FAH_{out}) = 10^{(pK_a - pH_{out})}/(1 + 2 \times 10^{(pK_a - pH_{out})} + 10^{(pH_{in} - pH_{out})}) \quad (8)$$

If the internal pH is lower than the external pH (Figure 3B), $p(FA^-_{in})$ will be smaller than $p(FA^-_{out})$, causing a net transmembrane flow of FA^- through the transition $1 \rightleftharpoons 4$ of diagram 3, J_{14}. Since all the other transitions are fast, this will cause net proton movement across the membrane, mediated by FA, J_H^{FA}. We will now derive how J_H^{FA} is related to J_{14}. Let for an infinitesimal duration δt an amount of $\delta\xi$ flow through this transition. As the actual flow occurs, this will have decreased the amount of FA^-_{out} by $\delta\xi$. Because of the near equilibrium of transitions $4 \rightleftharpoons 3$, $3 \rightleftharpoons 2$, and $2 \rightleftharpoons 1$, this decrease will immediately redistribute over all the forms of the fatty acid, such that, for instance

$$\delta_1 p(FAH_{in})/\delta\xi = -10^{(pK_a - pH_{out})}/(1 + 2 \times 10^{(pK_a - pH_{out})} + 10^{(pH_{in} - pH_{out})}) \quad (9)$$

Immediately after the transition, FA^-_{in} will have increased by $\delta\xi$, but also this increase will redistribute over all the forms of the FA, such that, using eqs 4 and 7, for instance

$$\delta_2 p(FAH_{in})/\delta\xi = +10^{(pK_a - pH_{out})}/(1 + 2 \times 10^{(pK_a - pH_{out})} + 10^{(pH_{in} + \delta pH_{in} - pH_{out})}) \quad (10)$$

where it should be noted that the internal pH may have changed by δpH_{in}. Because of the transition $4 \rightleftharpoons 3$, protons have been taken up from the internal volume:

$$-\delta H^+_{FA}/(N\,\delta\xi) = 1 + [\delta p(FAH_{in})/\delta\xi + \delta p(FAH_{out})/\delta\xi + \delta p(FA^-_{out})/\delta\xi - \delta p(FA^-_{in})/\delta\xi] \quad (11)$$

where the subscript FA to δH^+ stresses that this is the number of protons binding to the fatty acid. Using that

$$\lim_{\delta\xi \to 0} (\delta pH_{in}) = 0 \quad (12)$$

For instance

$$\lim_{\delta\xi\to 0}(\delta p(FAH_{in})/\delta\xi = (\delta_1 p(FAH_{in}) - \delta_2 p(FAH_{in}))/\delta\xi = 0 \quad (13)$$

Thus, all terms within the square brackets of eq 11 approach zero for $\delta\xi \to 0$, and the above equations lead to

$$J_H^{FA} = -d(H_{FA}^+)/dt = N\,d\xi/dt = NJ_{14} \quad (14)$$

This equation is similar to the equation that would have been derived for the well-known case where the number of excess protons inside the SUV greatly exceeds the number of FA molecules (i.e., the steady-state approximation). Because that assumption is not valid for our experiments, it was necessary to rederive the equation here.

Using diagram 3, we write for J_{14}

$$J_{14} = k_{FA^-}p(FA_{out}^-) - k'_{FA^-}p(FA_{in}^-) \quad (15)$$

Assuming membrane symmetry and the absence of a transmembrane electric potential, k_{FA^-} and k'_{FA^-} may be taken equal. Since during the experiments of Figure 3B, $pH_{in} - pH_{out} \ll 1$, $p(FA_{out}^-) - p(FA_{in}^-)$ can be approximated by the first term of the Taylor expansion:

$$p(FA_{out}^-) - p(FA_{in}^-) \cong [\ln(10)/(2(1 + 10^{(pK_a - pH_{out})}))](pH_{out} - pH_{in}) \quad (16)$$

Combining eqs 15 and 16, we find

$$J_{14} = k_{FA^-}[\ln(10)/(2(1 + 10^{(pK_a - pH_{out})}))](pH_{out} - pH_{in}) \quad (17)$$

Using the relationship between the change in internal pH and the number of protons added to the internal volume that defines the buffer capacity B_{in}, we find

$$B_{in}\,d(pH_{in})/dt = J_H^{FA} + J_H^b = NJ_{14} + J_H^b = [Nk_{FA^-}\ln(10)/(2(1 + 10^{(pK_a - pH_{out})})) + L](pH_{out} - pH_{in}) \quad (18)$$

By integrating this equation, we find a negative exponential approach of pH_{in} toward pH_{out} with relaxation constant

$$k_t = k_b + k_{FA^-}[N\ln(10)/(2(1 + 10^{(pK_a - pH_{out})}))]/B_{in} \quad (19)$$

Thus, the pseudo-unimolecular rate constant of flip of ionized FA, k_{FA^-}, can be calculated from the measured k_t and k_b in Figure 3B.

Noncyclic Proton Transport by FA. When FA (or bile acid) insert into the outer leaflet of vesicles, the un-ionized species will flip across in response to their concentration gradient and a fraction of these will deprotonate immediately to restore the ionization equilibrium in the inner leaflet. The rate of the acidification of the inner volume will depend on the rate of the influx of the un-ionized species. Assuming that in this time frame both the "background" proton leak and the flip of the ionized form are negligible (i.e., the ionophore valinomycin is not present), the initial rate at which protons appear in the inner volume, after N mol of FA (per mole of PC) has inserted in the outer leaflet, is

$$J_H^{t=0} = N\,d(p(FA_{in}^-))/dt \quad (20)$$

Assuming rapid equilibrium between the ionized and un-ionized FA at the inner leaflet (see eq 4), the following expression can be found:

$$d(p(FA_{in}^-))/dt = [1/(1 + 10^{(pK_a - pH_{in})})]d[p(FAH)_{in} + p(FA_{in}^-)]/dt \quad (21)$$

Combining eqs 20 and 21 yields

$$J_H^{t=0} = N[1/(1 + 10^{(pK_a - pH_{in})})]d[p(FAH_{in}) + p(FA_{in}^-)]/dt \quad (22)$$

At $t = 0$, all FA resides at the outer leaflet; i.e.

$$p(FA_{in}^-) = p(FAH_{in}) = 0 \quad (23)$$

$$p(FA_{out}^-) + p(FAH_{out}) = 1 \quad (24)$$

Using diagram 3, and neglecting the influx of ionized FA, we find at $t = 0$

$$d[p(FAH_{in}) + p(FA_{in}^-)]/dt = k_{FAH}p(FAH_{out}) \quad (25)$$

The combination of eqs 22 and 25 leads to

$$J_H^{t=0} = N[1/(1 + 10^{(pK_a - pH_{in})})]k_{FAH}p(FAH_{out}) \quad (26)$$

Assuming also equilibrium between the ionized and un-ionized form at the outer leaflet, we can, by combining eqs 5 and 24, express $p(FAH_{out})$ in terms of pH_{out}:

$$p(FAH_{out}) = 1/(1 + 10^{(pH_{out} - pK_a)}) \quad (27)$$

Combining eqs 26 and 27 yields

$$J_H^{t=0} = k_{FAH}N[1/(1 + 10^{(pK_a - pH_{in})})][1/(1 + 10^{(pH_{out} - pK_a)})] \quad (28)$$

$J_H^{t=0}$ can be measured following the changes in internal pH with the pyranin fluorescence, assuming

$$J_H^{t=0} = -B_{in}(d(pH_{in})/dt)^{t=0} \quad (29)$$

Thus, the pseudo-unimolecular rate constant of flip of un-ionized FA, k_{FAH}, (or un-ionized cholic acid, k_{CAH}) can be calculated from the initial rate of acidification in the experiments of Figures 5D and 8A by combining eqs 28 and 29:

$$k_{FAH} = -(d(pH_{in})/dt)^{t=0}(B_{in}/N)(1 + 10^{(pK_a - pH_{in})})(1 + 10^{(pH_{out} - pK_a)}) \quad (30)$$

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