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pH gradients across phospholipid membranes caused by fast flip-flop of un-ionized fatty acids

(transbilayer movement/phospholipid bilayer/serum albumin/pyranin/fluorescence)

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ABSTRACT **A central, unresolved question in cell physiology is how fatty acids move across cell membranes and whether protein(s) are required to facilitate transbilayer movement. We have developed a method for monitoring movement of fatty acids across protein-free model membranes (phospholipid bilayers). Pyranin, a water-soluble, pH-sensitive fluorescent molecule, was trapped inside well-sealed phosphatidylcholine vesicles (with or without cholesterol) in Hepes buffer (pH 7.4). Upon addition of a long-chain fatty acid (e.g., oleic acid) to the external buffer (also Hepes, pH 7.4), a decrease in fluorescence of pyranin was observed immediately (within 10 sec). This acidification of the internal volume was the result of the "flip" of un-ionized fatty acids to the inner leaflet, followed by a release of protons from ≈50% of these fatty acid molecules (apparent pK_a in the bilayer = 7.6). The proton gradient thus generated dissipated slowly because of slow cyclic proton transfer by fatty acids. Addition of bovine serum albumin to vesicles with fatty acids instantly removed the pH gradient, indicating complete removal of fatty acids, which requires rapid "flop" of fatty acids from the inner to the outer monolayer layer. Using a four-state kinetic diagram of fatty acids in membranes, we conclude that un-ionized fatty acid flip-flops rapidly ($t_{1/2} \leq 2$ sec) whereas ionized fatty acid flip-flops slowly ($t_{1/2}$ of minutes). Since fatty acids move across phosphatidylcholine bilayers spontaneously and rapidly, complex mechanisms (e.g., transport proteins) may not be required for translocation of fatty acids in biological membranes. The proton movement accompanying fatty acid flip-flop is an important consideration for fatty acid metabolism in normal physiology and in disease states such as cardiac ischemia.**

Unesterified fatty acids are a key intermediate in lipid metabolism. In the well-oxygenated heart, fatty acids constitute the primary fuel, and the availability of fatty acids in plasma is a controlling factor in the rate of intracellular fatty acid utilization (1). Recently, fatty acids have also been found to have diverse biological activities, serving as second messengers (2), K^+ channel activators (3), inhibitors of the binding of plasma low density lipoproteins to receptors (4), and uncouplers of oxidative phosphorylation (5, 6). Fatty acids are either delivered to a membrane [e.g., from serum albumin (7)] or generated within it [e.g., by phospholipase A_2 (8)] in one leaflet only. Movement of fatty acids across the membrane may be essential for their utilization.

At present there is controversy as to the mechanism and rate of transbilayer movement of fatty acids in membranes (7, 9). Investigations with cell systems have led to conflicting conclusions, suggesting either rapid passive diffusion ["flip-flop" (10)] across the plasma membrane (11, 12) or protein-mediated transport (13–15), although the latter studies have not distinguished whether the protein(s) sequesters fatty acids from the plasma into the membrane or actually transports them across the membrane, or both.

Investigations with model membranes have also been inconclusive. In studies of H^+ conductance through black lipid membranes, Gutknecht (16) concluded that fatty acids are poor protonophores because of very slow transbilayer movement of ionized fatty acids. The un-ionized form of fatty acids was presumed to flip-flop readily, but the presence of substantial amounts of organic solvent in such model membranes could greatly reduce the permeability barrier of un-ionized fatty acids (16). Using phospholipid vesicles as model membranes, Doody *et al.* (17) concluded that the flip-flop of fluorescently-labeled fatty acids was more rapid than transfer between vesicles, whereas Storch and Kleinfeld (18) reached the opposite conclusion. Moreover, the latter study suggested that the rate of flip-flop was faster for ionized than for un-ionized fluorescently-labeled fatty acids (18), contrary to what might be expected (16, 19–21). Native long-chain fatty acids are completely removed from vesicles by albumin within seconds (22) or minutes (19), suggesting flip-flop of fatty acids within the time resolution of these measurements. However, the latter approaches were unable to prove that fatty acids were present in both leaflets of the vesicles prior to the onset of the transfer process and unable to distinguish flip-flop of un-ionized and ionized species.

Our goal was to determine (*i*) whether fatty acids can flip-flop across a phospholipid bilayer rapidly enough for biological utilization (within seconds) and (*ii*) which form of fatty acids (un-ionized or ionized) crosses the bilayer more readily. We have used native long-chain fatty acids and small unilamellar vesicles (SUVs) composed of egg yolk phosphatidylcholine (PtdCho), with and without cholesterol, as a protein-free model membrane. Pyranin, a water-soluble fluorescent pH probe, was trapped inside the vesicles to measure the internal pH (pH_{in}) (23, 24). We hypothesized that fatty acids added to the outside of the vesicles would cross the PtdCho bilayer preferentially in the un-ionized form and produce a measurable decrease in pH_{in}. Our findings validate this hypothesis and elucidate features of fatty acid movement across bilayers.

MATERIALS AND METHODS

SUVs were prepared by sonication as before (19), except that 0.5 mM pyranin was present in 100 mM Hepes/KOH buffer at pH 7.4. Large unilamellar vesicles (LUVs) were prepared by extruding a hydrated egg PtdCho (Avanti Polar Lipids) suspension 19 times through an extrusion apparatus (25). Untrapped pyranin was removed by placing 1.0 ml of the SUV or LUV suspension on a gel filtration column (20 ml of Sephadex G-25, medium grade), washing with 100 mM Hepes/KOH (pH 7.4), and using a UV lamp to monitor the

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Abbreviations: BSA, bovine serum albumin; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; PtdCho, phosphatidylcholine.

separation. Stock solutions of oleic acid (10 mM) and fatty acid-free bovine serum albumin (BSA, 1 mM) (fraction V, Sigma) were made as before (19).

PtdCho vesicles were added to 2.0 ml of 100 mM Hepes/KOH buffer (pH 7.4) in a stirred cuvette placed in a Perkin–Elmer fluorimeter at ≈20°C [final concentration of PtdCho, 0.5 mg/ml (0.65 μmol/ml)]. The pH outside the vesicles (pH_{out}) was measured by a pH minielectrode (Microelectrodes, Londonderry, NH), and pH_{in} was measured by the pyranin fluorescence (excitation wavelength, 455 nm; emission wavelength, 509 nm). Time resolution (10 sec) was limited by mixing of the chemicals in the cuvette and the response time of the fluorimeter. To calibrate the dependency of fluorescence on internal pH, the vesicles were permeabilized for protons with nigericin (1 μg/mg of PtdCho), so that $pH_{in} = pH_{out}$ at all times (23), and the fluorescence was recorded as a function of pH. Fluorescence increased sigmoidally with higher pH, with an almost linear region from pH 6.8 to pH 7.8, as found previously (23, 24).

RESULTS

To test for proton transport that occurs exclusively by transbilayer movement of fatty acids, it was first necessary to ensure that the vesicles were sufficiently impermeable to protons in the absence of added fatty acids (23, 26). An aliquot of KOH was added to SUVs with trapped pyranin at pH 7.4 (Fig. 1*A*). pH_{out} increased from 7.40 to 7.73, but the fluorescence of pyranin (and therefore pH_{in}) changed very slowly, indicating slow leakage of H^+/OH^- across the bilayer ($t_{1/2} \geq 30$ min). Proton leakage was limited by the buildup of a diffusion potential balancing the flux (26) and could be increased significantly ($t_{1/2} \approx 5$ min) by addition of valinomycin (1 μg/mg of PtdCho), which collapsed the diffusion potential by counterflow of K^+ (data not shown). Upon addition of nigericin to make the bilayer permeable to H^+/OH^-, pH_{in} immediately increased to 7.73 (Fig. 1*A*).

To monitor movement of fatty acids from the outer to the inner leaflet of a phospholipid vesicle ("flip"), oleic acid was added to a buffered solution ($pH_{out} = 7.38 \pm 0.03$) of vesicles with entrapped pyranin ($pH_{in} = 7.35 \pm 0.03$). Fig. 1*B* shows the response of pyranin fluorescence to addition of 20 nmol of oleic acid to SUVs. As indicated by the drop in pyranin fluorescence, pH_{in} decreased by 0.22 unit within the 10-sec time resolution of the measurement. After the rapid pH decrease, the fluorescence of pyranin increased very slowly (0.02 pH unit in 30 min). Thus, proton leak was not enhanced by the presence of 1.5 mol % oleic acid in the PtdCho vesicles. When the experiment was done in the presence of valinomycin (1 μg/mg of PtdCho), a similar instant acidification of pH_{in} upon addition of oleic acid was found (as in Fig. 1*B*), followed by a faster equilibration ($t_{1/2} \approx 3$ min) of the pH gradient because of higher proton leakage (data not shown).

Fig. 1*B* shows that the "flip" of un-ionized oleic acid across the bilayer is fast. To demonstrate that movement in the reverse direction ("flop") is also fast, BSA was added to extract the oleic acid from SUVs. In the experiment shown in Fig. 1*C*, oleic acid (20 nmol) was added to SUVs, causing pH_{in} to drop to 7.13 (as in Fig. 1*B*). Before significant equilibration of the pH gradient occurred, aqueous BSA (10 nmol) was added, and an immediate increase of pH_{in} to 7.38 was found (Fig. 1*C*). This result shows that all fatty acid was removed from the vesicles and that fatty acid on the inner leaflet removed protons from the inside of the vesicle in order to "flop" to the outer leaflet (see *Discussion*).

Using the protocol in Fig. 1, we obtained the same results when (*i*) oleic acid was added to the vesicles as aqueous K^+ oleate or in ethanol solution, (*ii*) palmitic or stearic acid was substituted for oleic acid, (*iii*) SUVs containing 20 mol % cholesterol were used, (*iv*) LUVs [average diameter of ≈1000 Å (25)] were used in place of SUVs [average diameter of 250 Å (10)], (*v*) the experiments were done at pH 7.2 or pH 7.8, and (*vi*) oleic acid was added up to 7 mol % with respect to phospholipid. In the last case, the greater proportions of oleic acid produced larger pH changes inside vesicles, as expected. Additional control experiments excluded any immediate effects, other than internal pH changes, of oleic acid or BSA on the fluorescence of pyranin. (*a*) When vesicles were permeabilized for protons with nigericin (1 μg/mg of PtdCho), addition of oleic acid and/or BSA at concentrations similar to those used in Fig. 1 did not induce pH changes inside SUVs. Thus, binding of oleic acid to SUVs *per se* did not affect the pyranin fluorescence. (*b*) Pyranin (5 μM) was dissolved in 50 mM potassium phosphate buffer at pH 11. Addition of oleic acid (up to 20 μM), which is fully ionized and remains dissolved at this pH, had no effect on the fluorescence. (*c*) BSA (29 μM) with or without bound oleic acid did not show measurable fluorescence at the excitation and emission wavelengths used in these experiments.

Our experimental procedure was used also to model the biological role of serum albumin, which is both to donate fatty acids to, and to extract fatty acids from, membranes (1, 7).

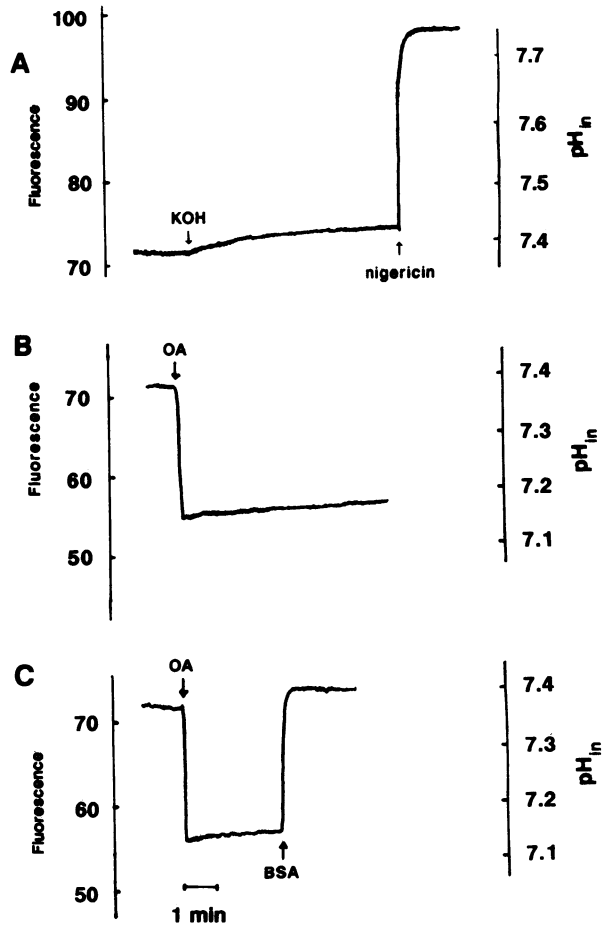

FIG. 1. Rapid flip-flop of un-ionized fatty acids in vesicles. (*A*) Addition of KOH, followed by nigericin, to SUVs with trapped pyranin. pH_{out} was measured with a pH electrode, and the pH_{in} by the fluorescence of pyranin. At the beginning, $pH_{in} = pH_{out} = 7.4$. Upon KOH addition, a pH gradient of 0.35 unit was established, which relaxed very slowly until nigericin was added, when $pH_{in} = pH_{out} = 7.73$. (*B*) Effect of adding 20 nmol of oleic acid (OA) (1.5 mol % with respect to PtdCho) as potassium oleate on the pyranin fluorescence of SUVs. pH_{in} decreased by 0.22 unit. pH_{out} remained at 7.38. (*C*) Addition of OA, followed by BSA, to vesicles with trapped pyranin. Addition of 20 nmol of OA caused pH_{in} to drop from 7.35 ± 0.03 to 7.13 ± 0.03. Addition of 10 nmol of BSA 3 min later instantly increased pH_{in} to 7.38 ± 0.03.

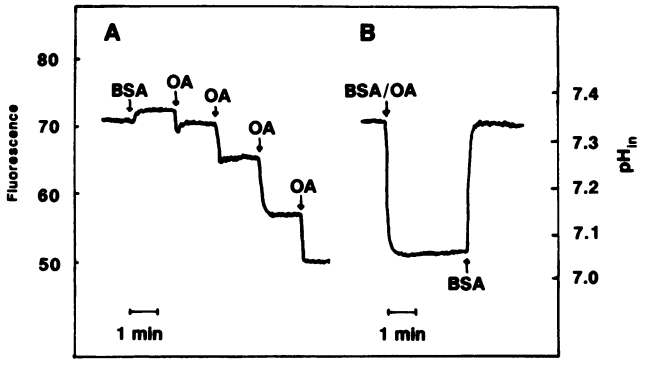

FIG. 2. Competition of binding of oleic acid (OA) to BSA and SUVs at various molar ratios of OA to BSA (*A*). BSA (10 nmol) was first added to SUVs, and a small increase in pH_{in} occurred as a result of extraction of endogenous fatty acids from the vesicles. Subsequently, four aliquots of 20 nmol of OA were added. Each addition was made before the pH gradient relaxed significantly. (*B*) BSA (10 nmol) loaded with OA (8 mol per mol of BSA) was added to SUVs. Delivery of OA to vesicles caused an instant drop in pH_{in}. Subsequent addition of 30 nmol of fatty acid-free BSA resulted in complete extraction of fatty acids, as reflected by an instantaneous increase in pH_{in} to the initial value before addition of fatty acids.

The partitioning of fatty acids will depend on the relative affinities of binding sites on albumin and membranes. Our procedure allows partitioning to be monitored without the need to separate any of the components. In the experiment illustrated in Fig. 2*A*, oleic acid was added in the presence of both albumin and SUVs. After the first addition of oleic acid (20 nmol), pH_{in} decreased only slightly, in contrast to the pH decrease of 0.22 unit observed after addition of 20 nmol of oleic acid in the absence of BSA (Fig. 1*B*). Thus, at a low molar ratio of oleic acid to BSA (2:1), some oleic acid partitioned to the vesicles but most was bound to albumin. Subsequent 20-nmol additions of oleic acid (which changed the molar ratio of oleic acid to BSA from 2:1 to 8:1) led to increased partitioning to the vesicles. When the oleic acid was loaded onto albumin in the total amount as in Fig. 2*A* (8 mol of oleic acid per mol of BSA) and then added to SUVs (Fig. 2*B*), pH_{in} dropped immediately to 7.05, indicating the same equilibrium distribution as in Fig. 2*A*. Subsequent addition of a large proportion of fatty acid-free BSA extracted all of the oleic acid (Fig. 2*B*). These results show that under conditions in which the three high-affinity binding sites on BSA are filled (27), the additional oleic acid bound to lower-affinity sites (7) readily partitions to the lipid bilayer (19, 28). Significantly, Fig. 2 shows that fatty acids move from albumin into, *and across*, the membrane quickly without involvement of additional protein(s) to sequester and/or transport them.

DISCUSSION

Our explanation of the above results is schematized in Fig. 3. Long-chain fatty acids added to the vesicle suspension at pH 7.4 bind rapidly and quantitatively to the phospholipid vesicles (28), initially to the outer leaflet (Fig. 3*A*). Since the apparent pK_a of oleic acid in egg PtdCho vesicles is 7.6 (19), the bound fatty acid consists of nearly equal amounts of un-ionized and ionized forms. Un-ionized fatty acids flip to the inner leaflet in response to the concentration gradient of fatty acid in the bilayer (Fig. 3*B*). At the pH of the inner volume, these fatty acids become about 50% ionized, releasing protons that diffuse to the inner aqueous volume containing pyranin (Fig. 3*C*).

Since the internal volume of SUVs is very small, ionization of a small quantity of fatty acid molecules at the inner monolayer will produce a measurable pH change. Taking the vesicle diameter to be 250 Å (10) and the cross-sectional area of a single PtdCho molecule to be 0.7 nm^2 (29), we calculate the internal volume to be ≈0.4 μl/mg of PtdCho. The buffer equation predicts that under the chosen experimental conditions, transport of 5 nmol of protons across the SUV membrane will cause a pH decrease from 7.38 to 7.12 in the inner volume. In Fig. 1*A* we observed a similar decrease following addition of 20 nmol of oleic acid, ≈5 nmol (25%) of which could donate a proton to the inner volume (Fig. 3*C*).

Addition of fatty acids to the external volume of vesicles established rapid movement of un-ionized fatty acids to the inner monolayer of the vesicles. Rapid movement in the reverse direction was demonstrated by addition of BSA to the external buffer. It has been previously shown that fatty acids desorb from a PtdCho bilayer and bind to albumin within seconds (28). Thus, when albumin extracts fatty acids from the external leaflet, un-ionized fatty acids "flop" from the inner to outer monolayer rapidly to restore the concentration equilibrium in the bilayer. The remaining ionized fatty acids on the inner monolayer bind protons from the internal aqueous compartment to reequilibrate to ≈50% ionization. Eventually, all fatty acids from the inner leaflet are extracted, and protons are removed from the inner aqueous volume, resulting in an increase in pH_{in}, as in Fig. 1*C*. The importance of our experimental design is that it showed that fatty acids were present in the inner monolayer prior to the addition of albumin to the external buffer.

Fig. 3*D* summarizes our proposed mechanism for fatty acid movement in a membrane in the form of a kinetic diagram (16, 23, 30, 31). The protonation and deprotonation reactions are assumed to be extremely fast, as for protonophores (30). Our

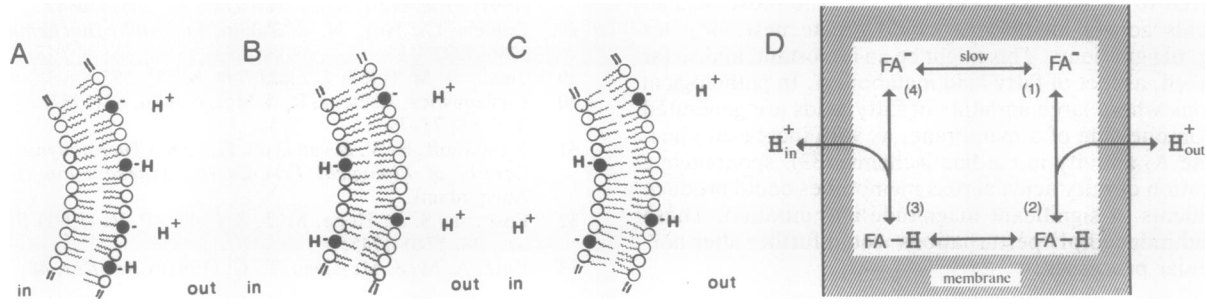

FIG. 3. Schematized mechanism of fatty acid movement across bilayers. (*A*) Four fatty acid molecules bound at the outer surface of a vesicle. Fifty percent of the fatty acid molecules are ionized. (*B*) The un-ionized fatty acids have flipped to the inner leaflet. (*C*) Fifty percent of the fatty acid molecules in both leaflets are ionized. The protons released inside the vesicle cause a measurable drop in pH. (*D*) Kinetic diagram. Fatty acid (FA) bound to the membrane of SUVs can be in four states: the head group can be ionized or un-ionized (protonated), at either the inner or the outer surface. The horizontal transitions represent the flip-flop reactions. The vertical transitions are the (de)protonation reactions. All transitions are reversible.

results show that the flip-flop of the un-ionized fatty acid is much faster than the flip-flop of the ionized fatty acid. If we assume a first-order process that is 90% complete within 10 sec, un-ionized fatty acids must flip-flop with $t_{1/2} < 2$ sec. The much slower rate of transbilayer movement of ionized fatty acids is crucial to our fluorescence measurements, which would not detect a pH decrease if the movement of un-ionized and ionized fatty acids occurred in a comparable time frame. One might argue that the instant acidification of pH_{in} upon addition of fatty acids to the external medium is the result of fast flip of ionized fatty acids as opposed to the un-ionized species. The acidification would then be the result of proton flux balancing the anion flux. This explanation can, however, be excluded, since the acidification was also found in the presence of K^+–valinomycin, when the charge compensation was ensured by K^+ flux. Furthermore, if the flip of the ionized species were faster than that of the un-ionized form, an instant alkalinization would have been observed under the latter conditions, which was not the case. Since a slow relaxation of the pH gradients of SUVs following the initial pH response was found (Fig. 1*B*), cyclic proton transfer of fatty acids must be slow ($t_{1/2} > 30$ min). In studies of fatty acid-mediated proton leak in planar membranes (16) the $4 \leftrightarrow 1$ transition (Fig. 3*D*) was also considered to be rate limiting. Our results for long-chain fatty acids are also consistent with results for other lipophilic acids [dicarboxylic acids (20), bile acids (21), and phosphatidic acid (32)] showing rapid transbilayer movement of the un-ionized (uncharged) form compared with the ionized (charged) form.

This study reveals fundamental properties of long-chain fatty acids in phospholipid bilayers, the structural framework of most cell membranes. The fast flip-flop of un-ionized fatty acids across phospholipid bilayers provides a simple, non-energy-dependent mechanism that satisfies the physiological requirement for rapid entry of fatty acids into, and their rapid removal from, cells such as adipocytes, hepatocytes, and heart muscle cells. Although our results do not disprove the existence of proteins that facilitate transbilayer movement of fatty acids in biomembranes, they show that the need for such a protein cannot be predicated on the assumption that a phospholipid bilayer necessarily constitutes a barrier to transbilayer movement of fatty acids.

The slow rate of flip-flop of ionized fatty acids in bilayers has important physiological implications as well. The inability of fatty acids to transfer protons cyclically across membranes should be considered in studies concerning the interference of fatty acids with oxidative phosphorylation (5, 6). Skulachev (6) has suggested that the ATP/ADP antiporter might enhance transbilayer movement of ionized fatty acids, enabling them to uncouple oxidative phosphorylation. Biological membranes have a low permeability to protons, and the maintenance of pH inside cells and cell organelles is considered to be well regulated (26, 31). The movement of fatty acids across membranes could create new, or alter existing, pH gradients. This might be an important, and so far overlooked, aspect of fatty acid metabolism. In pathological conditions where large amounts of fatty acids are generated locally on one side of a membrane, as with increased phospholipase A_2 activity in cardiac ischemia (33), spontaneous equilibration of fatty acids across membranes could produce pH gradients of significant magnitude and duration. These fatty acid-induced pH perturbations would further alter normal cellular processes.

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