A Mechanism for Tamoxifen-mediated Inhibition of Acidification*

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Tamoxifen has been reported to inhibit acidification of cytoplasmic organelles in mammalian cells. Here, the mechanism of this inhibition is investigated using in vitro assays on isolated organelles and liposomes. Tamoxifen inhibited ATP-dependent acidification in organelles from a variety of sources, including isolated microsomes from mammalian cells, vacuoles from Saccharomyces cerevisiae, and inverted membrane vesicles from Escherichia coli. Tamoxifen increased the ATPase activity of the vacuolar proton ATPase but decreased the membrane potential ($V_m$) generated by this proton pump, suggesting that tamoxifen may act by increasing proton permeability. In liposomes, tamoxifen increased the rate of pH dissipation. Studies comparing the effect of tamoxifen on pH gradients using different salt conditions and with other known ionophores suggest that tamoxifen affects transmembrane pH through two independent mechanisms. First, as a lipophilic weak base, it partitions into acidic vesicles, resulting in rapid neutralization. Second, it mediates coupled, electroneutral transport of proton or hydroxide with chloride. An understanding of the biochemical mechanism(s) for the effects of tamoxifen that are independent of the estrogen receptor could contribute to predicting side effects of tamoxifen and in designing screens to select for estrogen-receptor antagonists without these side effects.

Tamoxifen is the most commonly used treatment for breast cancer (1). In addition, it is currently being considered for widespread use in healthy women for breast cancer prevention (2, 3). Yet, despite its widespread use, its mechanisms of action remain obscure. Tamoxifen is a known estrogen receptor modulator that acts as an antagonist or partial agonist. But it has also been reported to have many pleiotropic effects both in vivo and in vitro that cannot be explained by an interaction with the estrogen receptor (4). For example, tamoxifen has been shown to enhance drug sensitivity of multidrug-resistant cells (5–9), inhibit bone resorption and osteoporosis both in vivo and in vitro (10), and inhibit a number of channels, including the volume activated chloride channel (11, 12) and calcium channels (13–16). These effects have been attributed to inhibition of P-glycoprotein (17), calmodulin (15), and direct channel interaction (11), respectively.

Previously, we have observed that tamoxifen inhibits acidification of intracellular organelles of both estrogen receptor positive and negative cell lines (18). This inhibition of acidification may be a mechanism for many of the effects of tamoxifen. For example, the effects of tamoxifen on osteoporosis (19), vesicular transport (20, 21), or multidrug resistance (9, 22) are mimicked by blocking the proton vATPase* or by a protonophore.

This work addresses the mechanism(s) by which tamoxifen inhibits ATP-dependent in vitro acidification of organelles isolated from tissue culture cells, whole tissue, vacuoles from Saccharomyces cerevisiae, and inverted vesicles isolated from Escherichia coli. The studies on yeast vacuolar acidification demonstrate that tamoxifen decreased both ATP-generated pH gradients and $V_m$ but increased the ATPase activity of the vATPase. These results suggest that tamoxifen affects ion permeability of a variety of biological membranes through interaction with either membrane proteins or the lipid bilayer.

The possibility that tamoxifen acts directly on the lipid bilayer was addressed with studies of pure lipid vesicles in which tamoxifen increased the rate of dissipation of the pH gradient. The data suggest that this occurs by two distinct mechanisms. First, tamoxifen is a lipophilic weak base with a neutral form that can readily flip-flop between membranes, and a basic form that is relatively impermeable. Thus, tamoxifen would accumulate in acidic vesicles, bind protons, and increase luminal pH. Importantly, tamoxifen is over 1000-fold more potent in increasing luminal pH than the soluble weak base ammonium chloride. This may be explained by the predominant partitioning of tamoxifen into the lipid phase, increasing the effective concentration. However, this mechanism can only be involved in dissipation of a pH gradient when the lumen is acidic. Second, tamoxifen can mediate coupled transport of proton or hydroxide with chloride based on the following observations: 1) it mediates electroneutral dissipation of pH gradients that is dependent on the presence of chloride or other halides; 2) it mediates an increased dissipation rate of chloride gradients; 3) it mediates net proton influx when the external chloride concentration is greater than the luminal chloride concentration.

Acidification is crucial for the proper functioning of many cellular processes, and its disruption may account for many of the pleiotropic effects described for tamoxifen. The results presented here show that at low micromolar concentrations, tamoxifen can inhibit acidification and dissipate pH gradients in a variety of in vitro systems, supporting in vivo data (18). Whereas this concentration is higher than required to modu-
late the estrogen receptor, it is similar to those reported for many estrogen receptor independent effects. Importantly, this concentration can readily be achieved in the clinic. The elucidation of a biochemical mechanism for this estrogen receptor independent activity of tamoxifen could significantly contribute to the design of modulators of the estrogen receptor that lack these side effects.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bafilomycin A1, monensin, acridine orange (AO), pyrane (8-hydroxypropyrene-1,3,6-trisulfonic acid), tamoxifen, Tris-ATP, and nigericin were from Sigma. BODIPY®-transferrin, lucigenin, pyrene-bispyridinium bromide (DPX) were from Molecular Probes (Eugene, OR). Adriamycin was from Calbiochem. Concanamycin A was from Fluka (Milwaukee, WI). Palmitoyloleoyl phosphatidylcholine (POPC) and cholesterol were from Avanti Polar Lipids (Alabaster, AL).

**Acidification of Cellular Microsomes**—Cells were grown in minimal essential medium supplemented with 10% fetal bovine serum to confluence in 10-level cell factories (Nunc, Naperville, IL), trypsinized, washed 3× with cold phosphate-buffered saline, and lysed with a Dounce homogenizer (pestle A) in 0.25 M sucrose, 20 mM HEPES, pH 7.4, 1 mM EDTA, 1× protease inhibitor mix (1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, and 16 μM phenylmethylsulfonyl fluoride) to make to 0.5 ml/ml (1×). The homogenate was centrifuged twice for 10 min at 3000 × g to remove unbroken cells and nuclei. The supernatant was layered over 20 ml of 0.5 M sucrose (20 mM HEPES, pH 7.4, 1 mM EDTA, 1× protease inhibitor mix) and 1 ml of 2 M sucrose and centrifuged for 1 h at 100,000 × g (Beckman Ti60 Rotor). Microsomes are collected at the 0.5 and 2 M interface.

To monitor acidification of the total microsomal fraction, the quenching of AO fluorescence was monitored essentially as described previously (23). Acidic vesicles accumulate AO to high concentrations resulting in the self-quenching of the dye and a decrease of the overall fluorescence. Fluorescence was measured on an SLM Aminco-Bowman series 2 luminescence spectrometer with λex = 488 nm and λem = 530 nm. Microsomes (80 μg of protein) were suspended in 2.5 ml of vesicle buffer (125 mM KCl, 5 mM MgCl2, 20 mM HEPES, pH 7.4, 1 mM EDTA, 1× protease inhibitor mix) and 6 μM AO (5 μM stock in H2O) in a cuvette. To examine the ability of vesicles to generate a ΔpH in the presence of tamoxifen or bafilomycin A1, 0, 1, 2, 4, or 8 μM tamoxifen (10 mM stock in DMSO) and 10 μM bafilomycin A1 (10 mM stock in 10% Me2SO) was added. After equilibration for 30 min at 25 °C, 1 mM Tris-ATP was added to begin acidification (100 mM stock, titrated to pH 7.4 with 1 mM Tris base before use). Acidification was monitored by excitation of the FITC fluorophore at 450 and 488 nm and measuring λem = 520 nm as described previously (18).

**Acidification of Yeast Vacuoles**—Vacuoles from S. cerevisiae were prepared from the protease-deficient strain B23407 (Yeast Genetic Stock Center, University of California, Berkeley) by sequential flotation through 12 and 8% Ficoll 400 cushion as described previously (24) with the single modification that 1× protease inhibitor mix and 1 mM EDTA was included in each step. This procedure produced a 25-fold enrichment of the vacular marker α-mannosidase (data not shown). Acidification was measured using AO as described above. For acidification in chloride-free solution, glutamate or glutamate was used in vesicle buffer instead of chloride.

**Acidification of E. coli Inverted Membrane Vesicles**—InV were prepared from the DH5α strain as described (25). Acidification was measured using AO as described above.

\[ V_{\text{m, vacuoles}} = \frac{V_{\text{m, vacuoles}}}{V_{\text{m, vacuoles}}} \]

**ATPase Activity of Yeast vATPase**—Vacuoles were diluted in KCl or potassium glutamate vesicle buffer. Each sample was split into two, and either 5 μM tamoxifen or carrier was added. Each of the four resulting samples was again split into two, and either carrier or 100 nM bafilomycin A1 was added. Next, 2 μM Tris-ATP was added to each sample, and the vacuoles were incubated at 30 °C for 15 min. To measure the phosphofructokinase concentration from ATP hydrolysis, an equal volume of 100 mM Tris-ATP, pH 7.3, with 1 μM tributyltin (TBT) a Cl– exchanger. This results in rapid net dissipation of the ΔpH. The fluorescence was monitored at 420 nm (see Fig. 5). The fluorescence was measured after each addition, and the pH was measured using a pH meter. The logarithm of the fluorescence ratio was linearly dependent on wall temperature overnight and then freeze-thawed 6 times. Unilamellar liposomes were prepared by extrusion 3 times through two stacked 100-nm Nucleopore (Corning/Costar Scientific, Acton, MA) polycarbonate membranes using an Avestin (Vancouver, British Columbia, Canada) extruder at 600 pounds/square inch. More than 95% of external pyranine was separated from the liposomes by sequentially running through NAP-10 and NAP-25 desalting columns (Amersham Pharmacia Biotech). Internal pyranine leakage was <1% per day, and liposomes were used within 1 week of preparation.

The pyramine fluorescence was calibrated as a function of pH by diluting the liposomes with pH 6.2 into a weakly buffered solution of identical pH (300 mM KCl, 1 mM MES, 1 mM MOPS, 1 mM Tricine, pH 6.2), 1 μM nigericin to allow rapid equilibration with external pH, and 5 mM DPX to quench external pyranine. The ratio of the fluorescence emission at λem = 510 nm was monitored with dual excitation wavelengths of λex = 405 and λem = 455 nm. Sequential aliquots of 0.1 mM glycylglycine, pH 8.4, were added to increase pH (see Fig. 5). The fluorescence was measured after each addition, and the pH was measured using a pH meter. The logarithm of the fluorescence ratio was linearly dependent on the pH. The curve generated by a least squares fit between pH 6.2 and 7.9 resulted in χ2 > 0.99. The calibration curve for the liposomes of luminal pH 8.1 was generated identically except sequential aliquots of 0.1 mM K-MES, pH 5.0, were added for titration, and the curve was generated between pH 8.1 and pH 6.4.

To measure the rate of pH dissipation of liposomes with luminal pH 6.2, the liposomes were diluted in weakly buffered solution of identical pH as described above but with no nigericin. Various agents (tamoxifen, valinomycin, and FCCP) were included as described in the text. The external pH was shifted to pH 7.3 by addition of 5 mM glycyglycine, pH 8.4, and the fluorescence ratio was monitored. After 10 min, 1 μM tamoxifen was added to dissipate the ΔpH, and the fluorescence was measured after each addition, and the pH was measured using the equation pH = xlog[AH+]/[A-] = 0.455 nm, where x and c are constants from the least square fit of the titration curve. To measure the rate of pH dissipation of liposomes with pH5 = 8.1, the identical procedure was followed except 5 mM K-MES, pH 5.0, was added to shift the external pH to 6.9.

To assay the effect of addition of NH4Cl or tamoxifen on lipidic pH, liposomes with pH5 = 6.2 were diluted into identical buffer (300 mM KCl, 20 mM MES, 20 mM MOPS, 20 mM Tricine, pH 6.2) containing 5 mM DPX, and the fluorescence ratio was followed after addition of NH4Cl or tamoxifen.

**Liposome Chloride Concentration**—Lucigenin is a fluorescent dye that is collisionally quenched by chloride and other halides but not by nitrate (26). Lipids dried as described above were rehydrated in 300 mM KNO3, 10 mM K-HEPES, pH 7.3, and 0.5 mM lucigenin. 100 mM unilamellar liposomes were made, and external dye was removed as described above.

To calibrate the fluorescence of lucigenin as a function of chloride, the liposomes were diluted in buffer (300 mM KNO3, 10 mM K-HEPES, pH 7.3) with 1 μM tributyltin (TBT) a Cl– exchanger, and 1 μM lucigenin was measured with a λex = 520 nm and λem = 530 nm. After fluorescence had equilibrated, vacuoles were added, and the fluorescence was allowed to re-equilibrate. Then, 1 mM Tris-ATP was added, and the resulting positive Vm was manifested in fluorescence quenching.

To measure the chloride permeability, the fluorescence was followed in liposomes after the addition of 50 mM KCl. After 10 min, 1 μM TBT was added.
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and 1 μM nigericin were added. The chloride concentration was calculated using the Stern-Volmer equation with k calculated from the titration curve.

Octanol Partitioning of Tamoxifen—The concentration of tamoxifen was measured by its absorbance peak at 245 nm. The A<sub>245</sub> of 20 μM tamoxifen in phosphate-buffered saline, HCl, pH 1, or KOH, pH 13, solution was acquired. Then, 1 μl of octanol was added, the solution was vortexed, and the A<sub>245</sub> of the aqueous phase was acquired.

RESULTS

In Vitro Acidification of Vesicles from Mammalian Cells

Total Microsomal Preparation—The mechanism by which tamoxifen inhibited acidification of intracellular organelles was first addressed by testing whether tamoxifen acted directly on the organelles or indirectly through soluble modulators. Acidification of organelles was assayed in vitro using microsomes isolated from MCF-7/ADR cells that are free of detectable soluble cytosolic proteins.

Acridine orange (AO) was used as a probe for luminal acidification (23). As vesicles acidify, they accumulate AO to self-quenching concentrations and deplete the extravesicular free AO, resulting in a decrease in total fluorescence. Acidification was initiated by the addition of ATP to a purified microsomal fraction in the absence of cytosol (Fig. 1A, inset). The ID<sub>50</sub> for maximal quenching is approximately 3 μM, which is in the same range that tamoxifen inhibits acidification in vivo (18). As a positive control, we employed bafilomycin A<sub>1</sub>, a potent and specific inhibitor of the vATPase responsible for acidification of all intracellular compartments (27).

To determine the time course for the inhibition of acidification by tamoxifen, the drug was added 10 min after addition of ATP (Fig. 1B). Addition of tamoxifen rapidly reversed acidification and caused an almost complete dissipation of the pH gradient within 5 min. Addition of bafilomycin A<sub>1</sub> dissipated the pH gradient at a much slower rate, even when used at 100 μM, which is 10 times the concentration that blocked 95% of the pH gradient at a much slower rate, even when used at 100 μM, solution was acquired. Then, 1 μl of octanol was added, the solution was vortexed, and the A<sub>245</sub> of the aqueous phase was acquired.

Mechanisms of Organelle Acidification

Acidification of intracellular organelles utilizes an electrogenic proton pump (the vATPase) and chloride channels (28, 29). The vATPase couples ATP hydrolysis to proton movement. The unidirectional movement of the proton generates an inside positive V<sub>m</sub> which limits acidification. The chloride channels allow passive chloride influx into the organelles, dissipating the V<sub>m</sub>. Tamoxifen could inhibit acidification by the following possible mechanisms: direct inhibition of the vATPase; indirect inhibition of the vATPase through modulation of the V<sub>m</sub> (such as blocking a chloride conductance); inhibition of acidification by a weak base effect or dissipation of pH gradients as a protonophore. There exists evidence in support of each of these mechanisms.

Inhibition of the vATPase—Tamoxifen has been reported to inhibit acid secretion by avian osteoclasts through inhibition of the plasma membrane vATPase activity. This activity has been attributed to the antagonism by tamoxifen of the membrane-bound calmodulin-dependent cyclic nucleotide phosphodiesterase, which regulates the vATPase (30).

Inhibition of the Chloride Channel—Tamoxifen has been reported to inhibit the volume-activated chloride channel (11).

Dissipation of pH Gradient by a Weak Base Effect—A weak base (such as ammonium chloride) will rapidly cross the membrane in a neutral (i.e. NH<sub>4</sub>) form and bind protons in the interior causing an alkaline shift. The charged form of these molecules will accumulate according to the Henderson-Hasselbach equilibrium. Tamoxifen is a weak base with a p<sub>Ka</sub> of 6.9 when measured by NMR in 10% Triton solution (31). At a free tamoxifen concentration of 8 μM, a pH 7.3–5.3 gradient will result in ~200 μM lumenal concentration. This is less than the buffering capacity of the organelles, and this should not significantly perturb lumenal pH. Thus, we initially considered this mechanism unlikely.

Dissipation of pH Gradient by Increasing Proton Permeability—Tamoxifen partitions into lipids, increases membrane fluidity, and decreases lipid peroxidation (32). If the charged protonated form of tamoxifen was membrane-permeable, tamoxifen would act like a classic protonophore. This mechanism has been proposed for the ability of many amine local anesthetics to uncouple respiration (33, 34).

Each of these potential mechanisms has distinct consequences for ATPase activity and V<sub>m</sub> of the acidic organelle (Table I). If tamoxifen inhibits the vATPase, it would decrease the ATPase activity. In addition, it should decrease V<sub>m</sub> of the organelles since the proton pumping is generating the V<sub>m</sub>. If tamoxifen inhibits the chloride channel, it would increase V<sub>m</sub> since the chloride channel serves to dissipate V<sub>m</sub>. As a consequence of the increased V<sub>m</sub>, the vATPase cannot pump protons, resulting in a decreased rate of ATP hydrolysis. If tamoxifen is a protonophore, it should decrease V<sub>m</sub> by allowing protons to permeate and increase ATPase activity by decreasing the electro-
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A. preincubation with tamoxifen. Acridine orange is a weak base that accumulates to self-quenching concentrations into acidic compartments. Thus, the presence of acidic compartments decreases the total fluorescence by decreasing the concentration of free AO outside those compartments. Microsomes were suspended in AO, and after establishing base line, 1 mM Tris-ATP was added to begin acidification (at 300 s). This caused a slow decrease of total fluorescence over 1200 s (Ctrl). Addition of the protonophore nigericin (5 μM Nig) at t = 1500 returned the fluorescence levels demonstrating the fluorescence decrease was the consequence of acidification. This inhibitory effect of tamoxifen (Tam) on acidification was apparent at 1 μM and increased in a dose-dependent manner (2, 4, and 8 μM). Pretreatment of microsomes with 10 nM bafilomycin A1 (Baf) also blocked acidification. Inset, dose-response of tamoxifen on acidification. Acidification was assayed as in A. B, tamoxifen added during the acidification. Ten minutes after the addition of 1 mM Tris-ATP, 8 μM tamoxifen or 100 nM bafilomycin A1 was added which rapidly reversed acidification of the organelles. In the absence of tamoxifen or bafilomycin A1, the organelles continued to acidify. Ten minutes later 5 μM nigericin was added. C, acidification in recycling endosomes assayed by FITC-transferrin. Cells were incubated with FITC-transferrin, which is endocytosed and localized within the endosomes. After lysing the cells a microsomal fraction was harvested. The fluorescence emission at 520 nm was monitored in response to excitation at 488 and 450 nm, and the ratio was plotted.

FIG. 1. Effect of tamoxifen on in vitro acidification of MCF-7/ADR organelles. A, preincubation with tamoxifen. Acridine orange is a weak base that accumulates to self-quenching concentrations into acidic compartments. Thus, the presence of acidic compartments decreases the total fluorescence by decreasing the concentration of free AO outside those compartments. Microsomes were suspended in AO, and after establishing base line, 1 mM Tris-ATP was added to begin acidification (at 300 s). This caused a slow decrease of total fluorescence over 1200 s (Ctrl). Addition of the protonophore nigericin (5 μM Nig) at t = 1500 returned the fluorescence levels demonstrating the fluorescence decrease was the consequence of acidification. This inhibitory effect of tamoxifen (Tam) on acidification was apparent at 1 μM and increased in a dose-dependent manner (2, 4, and 8 μM). Pretreatment of microsomes with 10 nM bafilomycin A1 (Baf) also blocked acidification. Inset, dose-response of tamoxifen on acidification. Acidification was assayed as in A. B, tamoxifen added during the acidification. Ten minutes after the addition of 1 mM Tris-ATP, 8 μM tamoxifen or 100 nM bafilomycin A1 was added which rapidly reversed acidification of the organelles. In the absence of tamoxifen or bafilomycin A1, the organelles continued to acidify. Ten minutes later 5 μM nigericin was added. C, acidification in recycling endosomes assayed by FITC-transferrin. Cells were incubated with FITC-transferrin, which is endocytosed and localized within the endosomes. After lysing the cells a microsomal fraction was harvested. The fluorescence emission at 520 nm was monitored in response to excitation at 488 and 450 nm, and the ratio was plotted. When excited at 488 nm, the fluorescence of FITC increases with increasing pH, but when excited at 450 nm, the fluorescence of FITC is pH-independent. Therefore, a decreasing ratio indicates acidification. Upon addition of ATP (t = 1080 s) there was acidification of the lumen of the microsomes as assayed by a decrease in the ratio of the 488:450 nm emission. Nigericin was added (t = 2500 s) to confirm that the fluorescent shift was the result of acidification. Successive additions of 2.5 μM tamoxifen caused alkalinization of endosomes. Nigericin was added at the end to equilibrate pH.

TABLE I

| Predicted effects of potential mechanisms of tamoxifen on $V_m$ and ATPase activity |
|----------------------------------|----------------|--------------|
| $V_m$                           | ATPase activity |
| Inhibit H-ATPase                | Decrease       | Decrease     |
| Block counter-ion transport     | Increase       | Decrease     |
| Increase proton permeability    | Decrease       | Increase     |
| Weak base effect                | Same to slight increase | Slight increase |

The predictions of these mechanisms were tested on isolated vacuoles from S. cerevisiae. Vacuoles from S. cerevisiae offer several advantages in biochemical studies of the actions of tamoxifen as follows. 1) They further address the specificity of the effects of tamoxifen (S. cerevisiae are known not to have an estrogen receptor). 2) They use the same basic machinery as mammalian organelles, a vATPase and chloride channel, to generate the proton gradient. 3) They can be purified in large amounts. It is very difficult to prepare mammalian organelles to the high purity required to assay $V_m$ and ATPase activity. In yeast vacuole preparations, the ATPase represents ~50% of all ATPase activity, which is much higher than attainable for endosome or Golgi preparations.

In Vitro Acidification of Yeast Vacuoles and E. coli Membrane Vesicles

Acidification of Yeast Vacuoles—To test if tamoxifen inhibited acidification in yeast vacuoles, in vitro acidification of vacuoles was assayed using AO. The buffer was pre-equilibrated with either carrier (Me2SO), tamoxifen (2 μM or 8 μM), ammonium chloride (1 mM), or concanamycin A (10 mM), or in potassium glutamate instead of KCl buffer (Fig. 2A). ATP (1.5 mM) was added at 50 s to initiate acidification, and at 400 s nigericin (1 μM) was added to dissipate pH gradients. As observed in mammalian microsomes, tamoxifen shows a dose-dependent inhibition of acidification, with complete inhibition at 8 μM. This strongly implies that tamoxifen inhibits acidification independent of the estrogen receptor which is not found in yeast. Acidification was slightly inhibited by the weak base ammonium chloride (1 mM). This is 1000-fold greater than the concentration of tamoxifen required to achieve similar inhibition.

Addition of tamoxifen to pre-acidified vacuoles (at 250 s in Fig. 2B) resulted in a rapid alkaline step followed by slower alkalinization. The step is reminiscent of a weak base which rapidly establishes equilibrium across vesicles. Thus, a comparison was made of the effects of adding tamoxifen and the weak base ammonium to pre-acidified vacuoles (Fig. 2B). Addition of ammonium (1 mM) indeed caused a step alkalization. However, the vacuoles slowly re-acidify after addition of ammonium but continue to alkalinize after addition of tamoxifen.

Membrane Potential—The fluorescent dye Oxonol V was used to monitor $V_m$. Oxonol V contains two delocalized negative charges and is highly lipophilic. In the presence of vesicles with positive $V_m$, it accumulates in the lumen and inner leaflet, resulting in fluorescence quenching (35). Unlike AO, which exhibits quenched fluorescence in the presence of acidic vesicles regardless of the number of non-acidified vesicles present, Oxonol V will report an average $V_m$ for all vesicles. This necessitates the use of pure preparations, such as the yeast vacuoles. The $V_m$ was monitored in either KCl or potassium gluconate buffer (Fig. 3A). At 50 s, tamoxifen (10 μM) or carrier was added, and acidification was initiated by adding ATP (2.5 mM) at 200 s. A larger positive $V_m$ was generated in potassium...
Vacuoles were suspended in buffer (KCl except potassium glutamate labeled) containing AO in the presence of tamoxifen (2 or 8 μM). At 50 s, ammonium sulfate (1 mM) or tamoxifen (2 μM) was added. Each caused a step increase in AO fluorescence, indicating alkalinization. Subsequently, AO fluorescence continued to increase when tamoxifen was added but slowly decreased when ammonium sulfate was added.

Glucuronate than in KCl which implicates a chloride permeability in dissipating the \( V_m \) of the vacuoles. Tamoxifen significantly decreased the \( V_m \) generated by the vATPase. This suggests that tamoxifen may increase ion permeability thus decreasing the \( V_m \).

**ATPase Activity**—To assay specifically the vATPase activity, the effects of tamoxifen were quantified on the bafilomycin-inhibitable ATPase activity. Replacing chloride with gluconate decreased the bafilomycin-inhibitable ATPase activity (Fig. 3B), further confirming that chloride provided the counter-ion transport to dissipate the \( V_m \). Addition of tamoxifen caused an increase of ATPase activity in both conditions, with a more dramatic increase in gluconic buffer (Fig. 3B).

In summary, in yeast vacuoles tamoxifen inhibited ATP-dependent acidification, decreased \( V_m \), and increased bafilomycin-inhibitable ATPase activity. These observations are consistent with the hypothesis that tamoxifen increases membrane permeability to protons, either through direct lipid interaction or through proteins or modulators (Table I).

**Acidification of E. coli Inverted Membrane Vesicles**—To test the protein and lipid specificity of acidification inhibition by tamoxifen, the effects of tamoxifen on ATP-dependent acidification in E. coli inverted vesicles (InV) was assayed. Unlike mammalian or yeast vesicles, InV utilize the \( F_0F_1 \)-ATPase for acidification and are composed of different types of lipids, including an abundance of cardiolipin and a lack of sterols.

As shown in Fig. 4, the presence of tamoxifen inhibited acidification in InV with a similar dose dependence as observed in mammalian and yeast vesicles (Figs. 1A and 2A). Similarly, addition of tamoxifen to E. coli vesicles pre-acidified with ATP resulted in similar rates of alkalization (data not shown) as mammalian and yeast vesicles (Figs. 1A and 2A). These observations indicate that tamoxifen can dissipate pH gradients across a diverse spectrum of native biological membranes.

**Liposomes**

**pH Gradients**—The effect of tamoxifen on pH gradients was tested in pure lipid vesicles. This system was used both because the effects of tamoxifen on acidification were observed in diverse biological membranes and because the results on the ATPase activity were consistent with tamoxifen affecting membrane permeability to protons.

Liposomes were loaded with pyranine, a hydrophilic, non-permeable fluorescent pH indicator to assay proton permeability. The log of the ratio of the fluorescence emission of pyranine when excited at \( \lambda_{ex} = 405 \) nm and \( \lambda_{em} = 455 \) nm is linearly dependent on the pH (Fig. 5). Two steps were taken to ensure that only pH was measured and to permit the discrimination between dissipation of pH gradient and the lysis or dye leakage from liposomes. First, greater than 95% of external pyranine was removed by gel filtration. Second, the membrane-impermeable quencher DPF was added to the external solution (Fig. 5B) which effectively quenched all remaining non-luminal pyranine fluorescence.

To mimic the acidic lumen of organelles, liposomes were made with the pH buffer at 6.2. The pH was monitored while the external pH (at 50 s) was shifted to 7.3 (Fig. 6A) in
the presence tamoxifen (0, 0.5, or 2 μM). Nigericin (1 μM) was added at 700 s to dissipate pH gradients. In the absence of tamoxifen, the pH increased less than 0.2 pH units over the 10-min span. In the presence of tamoxifen, after a shift of external pH, there was a rapid step increase in pH. The proton permeability of liposomes after the step increase is difficult to compare with the control, since the pH gradient has decreased. Tamoxifen did not induce detectable leakage of pyranine, and in solution, tamoxifen does not affect pyranine fluorescence (data not shown).

The effects of tamoxifen on pH were contrasted with the effects of other pH perturbants with known mechanisms of action, specifically a protonophore (FCCP), a potassium ionophore (valinomycin), and a weak base (NH4Cl) (Fig. 6B). FCCP at saturating concentrations only slowly dissipated the pH gradient. This is because FCCP allows free movement of only protons. Thus proton efflux down its gradient generates a Vm, preventing further proton movement. The presence of valinomycin, a K+-selective ionophore which would dissipate Vm, caused a faster dissipation of the pH gradient than FCCP (Fig. 6C). Here, protons can efflux down the concentration gradient without generation of Vm. Thus, these liposomes were more permeable to protons than potassium. As expected, the combination of FCCP and valinomycin immediately dissipated the pH gradient (data not shown) compared with the effects of nigericin. The weak base NH4Cl caused a step alkaline shift, followed by a slow alkaline drift. Here, the alkaline shift is caused by the selective diffusion of the basic NH3 into the vesicles, whereas the acidic NH4+ is impermeable. Of the three agents tested, the effect of micromolar concentrations of tamoxifen is most similar to the effect observed at millimolar concentrations of NH4Cl; the step alkalization upon changing the external pH was similar, but the subsequent dissipation of pH was faster with 0.5 μM tamoxifen (Fig. 6A).

The potential contribution of a weak base effect in the mechanism of tamoxifen action was further explored using liposomes in the absence of a pre-existing pH gradient. The pH was monitored and tamoxifen (2 and 8 μM) or NH4Cl (5 mM) was added at 50 s. At 700 s, nigericin was added to dissipate the pH gradient. As expected, upon addition of the weak base NH4Cl, the non-protonated species (NH3) rapidly diffused into the liposomes, where it was protonated causing alkalization of the lumen (Fig. 7A). The pH gradient slowly dissipated by either leakage of H+ or NH4+. Similarly, addition of tamoxifen caused alkalization of liposomes, followed by more rapid pH equilibration (Fig. 7A). This suggests that like ammonia, tamoxifen-free base rapidly enters liposomes, causing a step alkaline shift in the lumen while the protonated tamoxifen is less permeable.

The observation that tamoxifen exerted similar effects to NH4Cl at 3 orders of magnitude lower concentration suggests that it may be highly concentrated within liposomes. The extent of lipid partitioning of tamoxifen was examined by measuring the partitioning coefficient of tamoxifen between octanol and aqueous buffer. Tamoxifen in aqueous solution was equilibrated with either 1:1000 or 1:100 volume of octanol, and the concentration of tamoxifen left in the aqueous phase was measured by absorbance (Fig. 7B). Notice that 1:1000 volume octanol was able to extract approximately 50% of tamoxifen in aqueous solution, suggesting that tamoxifen partitions 3 orders of magnitude greater into the lipid phase. Octanol partitioning was repeated with the aqueous phase buffered to pH 1 and pH 13 to examine possible differences in partitioning between the charged and neutral forms of tamoxifen, respectively. The same result was obtained at both pH values (data not shown). This is consistent with the difference in concentrations of NH4Cl and tamoxifen required for the same quantitative effect. It may also contribute to the observation that pyranine reports a lower pH in the presence of tamoxifen even after addition of nigericin (Fig. 7A). Tamoxifen could effectively give the liposomes a positive surface charge, which has been shown to effect pyranine fluorescence (36).

In both yeast vacuoles (Fig. 2B) and liposomes (Fig. 7A), addition of tamoxifen or ammonium caused similar pH jumps, but tamoxifen subsequently caused a more rapid equilibration of pH. This suggests that other mechanism(s) may contribute in addition to the weak base effect. In liposomes with an acidic lumen, the initial weak base alkaline jump is too large to allow an assessment of the rate of subsequent pH dissipation. Therefore, we tested the effects of tamoxifen and a weak base on liposomes with an alkaline interior pH of 8.1. The pH was monitored after shifting the external pH to 6.9 by the addition of 4 mM MES, pH 5, at 50 s (Fig. 8A). The pH gradient was dissipated more quickly with increasing concentrations of ta-
moxifen. In contrast, the effect of NH4Cl (10 mM) was indistinguishable from the control. Thus, the dissipation of pH by tamoxifen cannot be solely explained as a weak base effect.

To explore potential ionophoretic mechanisms, tamoxifen was compared with FCCP and valinomycin (Fig. 8B). Addition of FCCP did not substantially increase the rate by which the pH gradient was dissipated, presumably because proton leakage is limited by the $V_m$. This is substantiated by the observation that valinomycin caused a greater dissipation of the pH gradient than FCCP.

The observation that tamoxifen diminished the pH gradient faster than a saturating concentration of FCCP (Fig. 8B) implies that tamoxifen cannot be a pure protonophore. Any pure protonophore will, like FCCP, allow free proton movement but be limited by $V_m$. Importantly, when both tamoxifen and valinomycin were included, the effect was additive and not synergistic. This implies that tamoxifen mediated proton movement is electroneutral. If tamoxifen mediated an electrogenic process (e.g. pure protonophore), the dissipation of the $V_m$ by valinomycin would dramatically increase the effect of tamoxifen. For example, the presence of valinomycin allows FCCP to immediately dissipate any pH gradient.

**Chloride Permeability**—If tamoxifen mediates bi-directional electroneutral transport of protons, then a second ion must be co-transported. We first asked if this ion could be chloride. We examined the effect of tamoxifen on the influx of chloride into liposomes. Lucigenin, a fluorescent dye that is collisionally quenched by chloride, was employed (39). Liposomes were loaded with KNO3 buffer and lucigenin. The lumenal chloride concentration can be accurately calibrated by the fluorescence (data not shown).

Lumenal chloride concentration in the liposomes was monitored after addition of 50 mM KCl to the external solution (Fig. 9A). Tamoxifen caused a dose-dependent increase in the rate of chloride influx. Since chloride is more membrane-permeable than potassium, unidirectional chloride movement is also expected to be limited by $V_m$ (40). Indeed, the presence of valinomycin increased the rate of chloride equilibration. To test if tamoxifen was affecting chloride permeability solely by dissipation of the $V_m$, tamoxifen was added to liposomes in the presence of valinomycin. The presence of valinomycin allowed FCCP to immediately dissipate any pH gradient.

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![Fig. 6. Rate of pH equilibration of pH 6.3 liposomes in a pH 7.3 bath.](image)

Pyranine-loaded liposomes with pH 6.3 was diluted into KCl buffer of same pH and 10 mM DPX and one of the follow compounds: carrier, tamoxifen (Tam) (0.5 and 2 μM), NH4Cl (1 mM), FCCP (1 μM), or valinomycin (Val) (1 μM). At 20 s, 5 mM potassium glycylglycine, pH 8.4, was added to raise pH to 7.3. At 700 s, 1 μM nigericin (Nig) was added to equilibrate pH. A, with the presence of tamoxifen, the pH shift caused a rapid alkaline shift of the lumen. This cannot be due to lysis because there was no decrease of total fluorescence indicating lack of dye leakage into DPX-containing external buffer. Ammonium, at 2000× concentration, caused a similar alkaline jump. But the rate of pH dissipation was faster after the alkaline jump with tamoxifen than ammonium. B, FCCP and valinomycin each increased the rate of pH dissipation, but did not cause an alkaline jump upon change of external pH.

![Fig. 7. Basis for weak base effect of tamoxifen.](image)

A, effect on liposome pH by weak base addition. Pyranine-loaded liposomes with pH 6.3 were diluted into KCl buffer of same pH and 10 mM DPX. At 50 s, tamoxifen (Tam) (2 and 8 μM) or NH4Cl (5 mM) was added. At 700 s, nigericin (Nig) (1 μM) was added. Addition of tamoxifen or NH4Cl resulted in alkalization of the lumen, presumably due to selective influx and protonation of the uncharged species. Importantly, following the alkaline jump, the pH re-equilibrated much faster when tamoxifen was used, suggesting a tamoxifen-mediated proton permeability. B, octanol partitioning of tamoxifen. Tamoxifen (20 μM) in phosphate-buffered saline was mixed with 1:1000 or 1:100 volume of octanol. The tamoxifen concentration of the aqueous phase was determined using absorbance spectroscopy. Notice the 1:1000 volume octanol was able to extract approximately ~50% tamoxifen from the aqueous phase. This suggests that tamoxifen partitions 3 orders of magnitude into the lipid phase and is consistent with the potency of tamoxifen compared with ammonium in A.
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DISCUSSION

Tamoxifen inhibits ATP-dependent acidification in intact cells (18), mammalian organelles (Fig. 1), yeast vacuoles (Fig. 2), and InV (Fig. 4). It also dissipates pH gradients in liposomes (Figs. 6–9). The tamoxifen-dependent dissociation of the pH gradient is independent of all proteins including the estrogen receptor.

Our results suggest that tamoxifen affects transmembrane pH through at least two independent mechanisms as follows: as a weak base and as a mediator of coupled transport of proton/hydroxide and chloride. For vesicles with an acidified lumen, tamoxifen causes a rapid alkaline shift of the pH, which is most likely a weak base effect (Fig. 10). We propose tamoxifen is highly concentrated within the leaflets of membranes. Since tamoxifen is a weak base, its neutral form can readily flip between inner and outer leaflets while the charged form flips much less readily. Therefore, it will accumulate within the inner leaflet of acidic organelles, causing a step alkalinization.

However, a weak base effect is not sufficient to account for many of the effects of tamoxifen on transmembrane pH. Tamoxifen, but not ammonium, can increase pH equilibration rate when the lumen of the liposome is alkaline relative to the bath. Furthermore, chloride is necessary for tamoxifen-mediated proton permeability, and a chloride gradient can generate a pH gradient in the presence of tamoxifen. One possible mechanism for this process is that the permeability of the protonated form of tamoxifen increases when it is conjugated to chloride (Fig. 10).

The fact that tamoxifen inhibits acidification in so many model systems indicates that tamoxifen should affect organelar pH in many different cell types. This is consistent with the observations that tamoxifen administration has numerous physiological sequelae that are not restricted to cells expressing the estrogen receptor. Of particular significance is the observation that blocking organelle acidification through other means is sufficient to reproduce many of the effects of tamoxifen. Tamoxifen blocks bone resorption which is also blocked by antagonists of the vATPase (19). In drug-resistant tumor cells tamoxifen redistributes chemotherapeutics from the organelles to the cytoplasm (18) and increases the sensitivity of the cells to chemotherapeutics (41). These effects can be reproduced solely by mimicking the effects of tamoxifen on organelar acidification either with the use of protonophores, weak bases, or inhibitors of the vATPase (9, 22, 41). Tamoxifen decreases the rate of vesicle sorting and secretion (18) which is also seen when organelle acidification is blocked with protonophores.
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**REFERENCES**


**FIG. 10. Model for tamoxifen-mediated proton permeability.**

Tamoxifen is concentrated within the lipid bilayer and exists as a charged protonated form (TNH⁺) or uncharged form (TN). The uncharged form is readily membrane-permeable, and the charged form is impermeable. This accounts for the weak base activity of tamoxifen. Further, TNH⁺ can permeate the membrane when carrying a chloride ion, accounting for the chloride-dependent electroneutral proton permeability.

(42). Many secreted proteins are activated by a pH-dependent proteolytic step in the Golgi. Thus, the reduced activity of many secreted proteins observed with tamoxifen treatment may also be the consequence of a tamoxifen block of organelle acidification.

Consistent with previous reports (43), we observe that tamoxifen accumulates in the lipid phase (1000:1) over the aqueous environment. Furthermore, our results suggest that membrane-bound tamoxifen is in equilibrium between a neutral and protonated form. Thus, tamoxifen would be expected to significantly perturb many properties of cellular membranes, including increased surface charge, and altered membrane tension. These effects have been reported for lipophilic weak base anesthetics (44–46). The altered membrane properties could shift the voltage dependence of many ion channels. Indeed, tamoxifen has been reported to shift the activity of many ion channels (11–16). Detailed studies on the model channel, gramicidin, have shown that membrane tension (47) and surface charge (48) are critical determinants of channel activity.

These results demonstrate that many of the effects of tamoxifen on cells can be attributed to either membrane-active effects on organelle acidification or surface charge. Each of these effects are independent of the estrogen receptor. This suggests that it should be possible to screen for other estrogen-receptor antagonists that do not also affect organelar acidification and therefore may not share the same physiological effects as tamoxifen.

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**FIG. 9. Effect of chloride on tamoxifen-mediated proton permeability.**

A. Rate of chloride influx into liposomes. Lucigenin, a non-permeable fluorescent probe that is collisionally quenched by chloride, was used to assay liposome chloride concentration. Lucigenin-loaded liposomes made with an internal solution of 150 mM KNO₃ were diluted into 150 mM KNO₃ buffer and one of the follow compounds: carrier, tamoxifen (Tam) (2, 4, and 8 μM), valinomycin (Val) (1 μM), or both tamoxifen (4 μM) and valinomycin (1 μM). At 50 s, 50 mM KCl was added, and the internal chloride concentration was followed using lucigenin fluorescence. Tamoxifen caused a dose-dependent chloride influx. Valinomycin also increased chloride influx by dissipating Vₐₒₚ. Importantly, tamoxifen and valinomycin together were additive, implying that tamoxifen is not a pure chloride ionophore but mediates electroneutral chloride influx.

B. Effect of chloride on tamoxifen-mediated proton permeability. Pyranine-loaded liposomes made with internal solution of 300 mM potassium glutamate, pH 8.1, was diluted into the same external solution in the presence or absence of tamoxifen and 50 mM KCl as denoted in legend. At 50 s, pH₄ₒₐ was shifted to pH 6.9 and pH₄ₒₐ was followed. In the absence of chloride, tamoxifen had no effect on the rate of pH equilibration. The presence of chloride reconstituted the effect of tamoxifen seen in Fig. 8 C. Liposomes made as in B were diluted into 300 mM potassium glutamate solution. Addition of 4 μM tamoxifen caused an alkaline shift similar to Fig. 7A. But the rate of re-equilibration was much slower than in Fig. 7A. Addition of 2 aliquots of 50 mM KCl caused increasing rate of acidification, presumably due to Cl⁻/H⁺ co-influx. Nig, lucigenin.
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