

The Effects of Tamoxifen and Toremifene on Bone Cells Involve Changes in Plasma Membrane Ion Conductance

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ABSTRACT

Selective estrogen receptor modulators (SERMs), tamoxifen (Tam) and toremifene (Tor), are widely used in the treatment of breast cancer. In addition, they have been demonstrated to prevent estrogen deficiency-induced bone loss in postmenopausal women. These effects are thought to be caused by the interaction of the SERMs with the estrogen receptor, although SERMs have also been shown to conduct non-receptor-mediated effects such as rapid changes in membrane functions. We compared the effects of Tam, Tor, and 17β -estradiol (E2) on the viability of rat osteoclasts and osteoblasts. Both Tam and Tor were found to cause osteoclast apoptosis in *in vitro* cultures, which was reversed by E2. In addition, at higher concentration (10 μ M), both SERMs had an estrogen receptor-independent effect, which involved interaction with the plasma membrane as demonstrated with UMR-108 osteosarcoma cells by Tam and Tor, but not E2. A leak of protons leading to changes in intracellular pH was shown both in medullary bone derived membrane vesicles and in intact cells. These effects were followed by a rapid loss of cell viability and subsequent cell lysis. Our results show that both Tam and Tor have an ionophoric effect on the plasma membranes of bone cells and that these SERMs differed in this ability: Tor induced rapid membrane depolarization only in the presence of high concentration of potassium. These non-receptor-mediated effects may be involved in therapeutic responses and explain some clinical side effects associated with the treatment of patients with these SERMs. (*J Bone Miner Res* 2003;18: 473–481)

Key words: estrogen, tamoxifen, toremifene, osteoclast, plasma membrane

INTRODUCTION

ESTROGEN HAS AN important role in the development and growth of bones and later in the maintenance of the bone mass. Postmenopausal estrogen deficiency causes bone loss and leads to increased risk of fractures. Although the importance of estrogen is evident, the mechanisms behind the effects are not fully understood. It is well demonstrated that 17β -estradiol (E2) decreases differentiation of osteoclasts from the mononuclear precursors.^(1,2) We have recently shown that E2 affects the resorption activity of mature osteoclasts, especially by reducing the depth of resorption pits.⁽³⁾ E2 has also been suggested to inhibit bone resorption by inducing osteoclast apoptosis,^(4,5) although contradictory data have also been reported.^(6,7)

Selective estrogen receptor modulators (SERMs) are compounds that have both estrogen agonist and antagonist properties. They can function in the same way as estrogen in some tissues (e.g., bone) and like antiestrogen in other tissues (e.g., breast).⁽⁸⁾ The nonsteroidal SERM tamoxifen (Tam) and its chlorinated derivative toremifene (Tor) are used for the treatment of breast cancer,⁽⁹⁾ and their role in the prevention of breast cancer has also been surveyed.⁽¹⁰⁾ In addition, both Tam and Tor have been shown to have an effect on bone mineral density (BMD) in postmenopausal women.⁽¹¹⁾ Both SERMs bind to the estrogen receptor (ER), the interaction of which mediates many of the biological and chemotherapeutic effects of these compounds. However, additional non-ER-mediated mechanisms exist.⁽¹²⁾ The anti-cancer efficacy is partly based on the ability of these compounds to decrease proliferation of breast cancer cells. Tor⁽¹³⁾ and Tam⁽¹⁴⁾ have also been shown to induce

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programmed cell death, the latter even in ER-negative breast cancer cells.⁽¹⁵⁾

Several mechanisms of action for the ER -independent effects of the SERMs have been suggested. For instance, Tam has been shown to induce fast changes in intracellular calcium movements,^(16,17) and there is evidence of a direct interaction of Tam with protein kinase C,⁽¹⁸⁾ calmodulin⁽¹⁹⁾ transforming growth factor (TGF)- β ,⁽²⁰⁾ and P-glycoprotein.⁽²¹⁾ The interaction with P-glycoprotein is probably associated with the important ability of Tam and Tor to reverse the multidrug resistance syndrome.^(21,22) Recent studies have implicated the role of caspases⁽²³⁾ and mitogen-activated protein kinases.⁽²⁴⁾ Also oxidative stress⁽²⁵⁾ and changes in mitochondria function⁽²⁶⁾ may play important roles in TAM-induced apoptosis. Some earlier results have demonstrated that Tam can interact directly with membrane lipids⁽²⁷⁾ and change membrane properties such as fluidity.⁽²⁸⁾ This is important because most SERMs are highly lipophilic compounds that can accumulate in biomembranes and produce even high local concentrations. This is in agreement with the measurement of much higher concentrations of Tam in tissues than in serum.^(29,30) Special interest has been focused on the possibility that some of the side effects, such as cataract, in Tam treated patients⁽³¹⁾ are caused by other than ER -related effects. As shown by Zhang et al.,⁽³²⁾ Tam is able to block particular chloride channels, which might impair the maintenance of normal ionic concentrations and hydration of the lens. Accordingly, reduction in transmittance of bovine lenses have been observed in tissue culture in the presence of high concentration of Tam.⁽³³⁾

In this work, we have studied the effect of E2, Tam, and Tor in different bone cell models. We show that both Tam and Tor, but not E2, induced apoptosis in osteoclasts. Additionally, both Tam and Tor decreased the number of osteoblasts at high concentrations. This was studied in detail to characterize the possibility of direct, non-receptor-mediated effects of E2 and the SERMs in vitro. We show here that both Tam and Tor could directly interact with the plasma membrane and make it permeable to protons, although this effect was different in Tam and Tor. The changes in membrane permeability caused drastic changes in intracellular ion concentrations and membrane potential, which, at high Tam or Tor concentration, eventually led to cell death caused by the disintegration of the cell membrane.

MATERIALS AND METHODS

Reagents

All cell culture reagents, if not otherwise mentioned, were obtained from Life Technologies, Inc. (Grand Island, NY, USA). E2 (Sigma Chemical Co., St. Louis, MO, USA), Tam, and Tor (both from Orion Corp., Oulu, Finland) were dissolved either in ethanol or methanol as a 10 mM stock solution. The maximal amount of vehicle in cell cultures was 0.1% ethanol/methanol. Hoechst 33258 and leukocyte acid phosphatase kit 386-A were purchased from Sigma.

Osteoclast culture and apoptosis analysis

Mixed rat bone cell populations were cultured on bovine bone slices as previously described.⁽³⁴⁾ Osteoclasts were mechanically harvested from the long bones of 1- to 2-day-

old rats. Cells were cultured in phenol red-free Dulbecco modified Eagle's medium (DMEM) buffered with 20 mM HEPES and containing 0.84 g sodium bicarbonate/liter, 2 mM L-glutamine, 100 IU of penicillin, 100 μ g streptomycin/ml, and 10% heat-inactivated FCS for 24 h. Cells were stained with TRACP (Kit 386-A) and DNA-binding fluorochrome Hoechst 33258. Osteoclasts with fragmented nuclei and shrunken cytoplasm were counted as apoptotic.⁽³⁵⁾

Cell culture

UMR-108 rat osteosarcoma cells (ATCC CRL 1663; American Tissue Culture Collection, Rockville, MD, USA) were cultured in a phenol red-free RPMI-1640 medium, supplemented with 5% heat-inactivated FCS. The cells were grown in plastic tissue culture dishes (Nunc, Roskilde, Denmark) and subcultured twice a week. For experiments involving hormone or antihormone treatment, the growth medium was supplemented with FCS stripped twice with dextran charcoal (2 \times DC-FCS).

Determination of the cell number

UMR-108 cells were plated at a density of 1×10^5 cells/well in a 6-well plates (Nunc) and allowed to grow for 24 h in a phenol red-free RPMI-1640 medium supplemented with 5% 2 \times DC-FCS. Media were changed, and 1 and 10 μ M E2, Tam, or Tor were added to three parallel wells. Control cells received the vehicle only. The cells were counted in a Coulter Counter particle counter (Coulter Counter Ltd., Harpenden, UK) on days 1, 2, 3, and 4.

Preparation of the microsomal vesicle fraction

For the source of vacuolar H⁺-ATPase, we used osteoclast-enriched medullary bone. Bones were removed from regularly laying hens and homogenized in an Ultra-Turrax homogenizer in a homogenizing buffer containing 20 mM Tris-HCl pH 7.4, 250 mM sucrose, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM EGTA, and 1 mM dithiothreitol (DTT). The homogenate was centrifuged at 1000g for 15 minutes, and the pellet was rehomogenized and re-centrifuged. Combined supernatants were centrifuged at 10,000g for 15 minutes, and the resulting supernatant was centrifuged at 100,000g for 50 minutes. The final pellet (PIII) was suspended in the homogenizing buffer using a glass-Teflon homogenizer, frozen with liquid nitrogen, and stored for later use. Only frozen pellets were used in experiments.

Purification of phospholipid liposomes

About 5 g of bovine kidney was cut into small pieces, and 50 ml of chloroform-methanol-water (10:5:1) was added. The mixture was kept on ice for 60 minutes, shaken vigorously every 5 minutes. It was then centrifuged for 30 minutes at 5000g at 4°C. The chloroform layer was taken and evaporated. The solid lipids were dissolved in 3 ml of 20 mM Tris-HCl, pH 7.4, containing 200 mM n-octylglucoside. The mixture was then dialyzed overnight against 3000 ml of 20 mM Tris-HCl, pH 7.4.

Measurement of cytosolic pH

The UMR-108 cells cultured on glass coverslips were loaded for 30 minutes at 37°C with 0.5 mM 2',7'-bis-(2-carboxyethyl)-5-carboxyfluorescein-tetracetoxymethyl ester (BCECF-AM) in one of the following buffers. The normal buffer contained 127 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 0.5 mM NaH₂PO₄, 2 mM CaCl₂, 5 mM NaHCO₃, 10 mM glucose, and 10 mM HEPES, pH 7.2, whereas the high K⁺ buffer contained 127 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, and 10 mM HEPES, pH 7.2, and the K⁺-free buffer contained 127 mM choline chloride, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, and 10 mM HEPES, pH 7.2. Pluronic acid (0.02%; Molecular Probes, Inc., Eugene, OR, USA) was added to improve the loading. All dyes were dissolved in DMSO, which had no effect in any of the experimental conditions. After several washes, the coverslips were placed in a chamber on the stage of a Nikon TMD Diaphot inverted microscope surrounded by a incubation hood (Nikon, Tokyo, Japan), which was kept at 37°C. The exchange of buffer was performed using perfusion equipment providing gradual addition of different solutions. For measuring, the pH was adjusted to 7.8. The camera and image intensifier used were Sony CCD 72E (Dage-MTI Inc., Michigan City, MI, USA) and Videoscope KS-1381 (Videoscope International Ltd., Washington, DC, USA), respectively. Cells were excited at 495 and 440 nm for pH measurements by a computer-driven filter wheel (MAC 2000; Ludl Electronic Products Ltd., Hawthorne, NY, USA) and emitted light was obtained through a dichroic mirror and interference filters at 510 nm. The 495/440 ratios were obtained on a pixel-by-pixel basis and converted to pH values based on a calibration table for UMR-108 cells.

Intracellular recording of resting potential

The cells were placed in PBS in a shallow Petri dish. Membrane potentials were recorded by conventional techniques⁽³⁶⁾ using glass microelectrodes filled with 2 M potassium acetate and 5 mM KCl (pH 7.0 adjusted with PBS, KOH, and HCl). Input resistances of the electrodes were 40–70 MΩ. We used an electrode holder with a string attachment on the electrode side, made of chloridized silver wire (diameter, 0.3 mm), to soften the electrode impact on the cells. The cells were impaled either by small movements by a micromanipulator or light tapping of the vibration isolated table. All recorded electrical signals were amplified (Dagan 8100–1; Dagan Co., Minneapolis, MN, USA) and stored on DAT tapes (Biological DTR-1800; Biological Ltd., Claix, France). All recordings were monitored during recordings and reproduced using a digital oscilloscope with thermal printer facility (DL-1100; Yokogawa, Tokyo, Japan). The input resistance of the cells was monitored by passing current pulses through the recording electrode either in bridge or in switched-clamp mode⁽³⁷⁾ and recording the resulting voltage change.

Proton transport experiments

Proton transport by isolated membrane vesicles was measured as previously described.⁽³⁸⁾ When measuring the inhibitory effect, the compound studied was preincubated for

5 minutes with vesicles before adding the ATP. Proton transport activity was calculated from original curves by measuring the proton load for the first 2 minutes. When measuring the proton leak, protons were allowed to accumulate for 4 minutes, and subsequently the compound being tested was added to a cuvette. The affectivity of leakage was calculated from the original curves by measuring the proton leak for the first minute and comparing it to the total proton accumulation. The interaction of SERMs with phospholipid was determined by incubating them with liposomes for 1 minute before adding PIII vesicles and ATP. Tam, Tor, and E2 were dissolved in methanol just before measuring.

Statistical analysis

Differences between groups were assessed by ANOVA and Student's *t*-test. A *p* value <0.05 was considered significant. All columns describe the mean ± SE.

RESULTS

The effect of E2, Tam, and Tor on bone cells

We first studied the effect of E2 and the two SERMs on the viability of the mature osteoclasts using 24-h rat osteoclast cultures. At the concentrations used, E2 had no effect on the number of osteoclasts, on the number of apoptotic osteoclasts, or on the apoptosis index (Fig. 1A). An apoptosis index was counted as the number of apoptotic osteoclast per total number of osteoclasts × 100. Because serum withdrawal is known to induce apoptosis in osteoclasts, we next studied the effect of E2 on osteoclast apoptosis under serum-free conditions (results not shown). Serum deprivation as such caused an approximately 70% increase in the apoptosis index, and E2 partially protected osteoclasts from serum starvation-induced apoptosis. As shown in Fig. 1B, addition of Tam to culture medium at a low concentration of 1 nM induced a marked increase in osteoclast apoptosis. The effect of Tam was completely, and dose dependently, blocked by E2 (Fig. 1C) at low Tam concentration (1 nM). Tor also induced osteoclast apoptosis (Fig. 1D), but the effect was not as drastic as with Tam. At high concentration (10 μM), however, both Tam and Tor (Figs. 1B and 1D) dramatically reduced the number of osteoclasts, and at these high concentrations, E2 was not able to reduce the apoptosis rate (data not shown). Serum was present in all experiments shown in Fig. 1.

We next studied whether osteoblastic cells were similarly affected by E2 and the SERM compounds. Rat osteosarcoma cell line (UMR-108) cells were cultured for 4 days in the presence of E2, Tam, or Tor (Fig. 2A). E2, even at 10 μM concentration, did not disturb cell growth. Tam and Tor at 1 μM concentration slightly retarded the rate of cell growth. The addition of 10 μM Tam or Tor to the culture medium almost completely prevented cell proliferation. We next studied the effects of different Tam concentrations further. Figure 2B shows the effect of Tam on the nonosteoclastic cells included in the rat osteoclast-enriched bone marrow cell cultures. The number of mononuclear cells/1000 μm² from 10 separate locations at each bone slice was counted. We found that 10 μM Tam drastically reduced the cell number, but lower concentrations had no effect (except

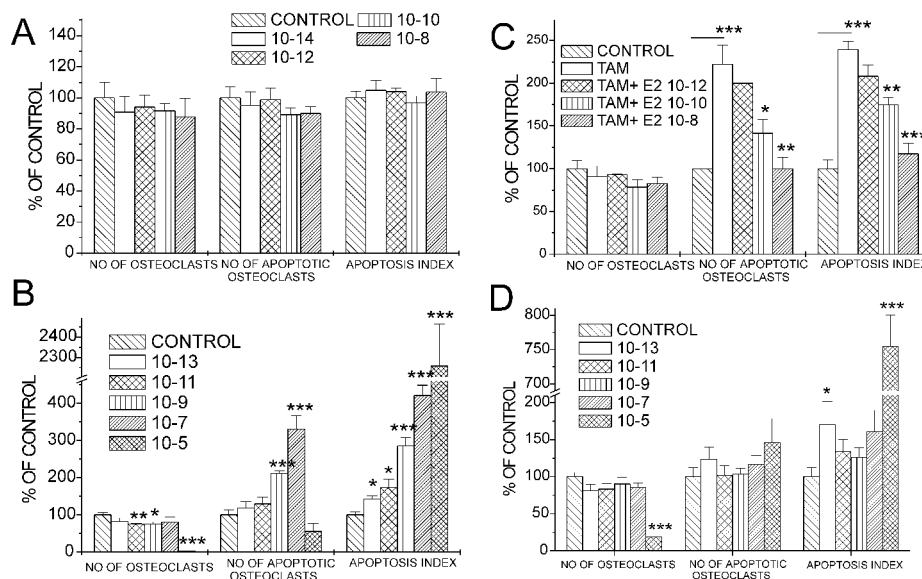


FIG. 1. The effect of (A) E2, (B) Tam, (C) Tam + E2, and (D) Tor on the number of normal and apoptotic osteoclasts after 24 h in culture. Cell numbers are presented as percentage of control. Apoptosis index was counted as number of apoptotic osteoclasts/total number of osteoclasts. Absolute numbers for control groups are 165.25 ± 16.72 , 165.00 ± 10.21 , 50.25 ± 4.94 , and 150.70 ± 29.20 osteoclasts for E2-, Tam-, Tam + E2-, and Tor-treated groups, respectively. The average number of apoptotic osteoclasts/bone slice in control groups was 21.77 ± 3.47 and the average apoptosis index in control groups was 17.88 ± 2.90 . The p values from Student's t -test between control and other groups are shown as asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). In C, the asterisks showing the statistical significance between the control and Tam-treated group have been underlined. Other asterisks in C show the comparison between Tam- and Tam + E2-treated groups. The concentration of Tam used in this experiment was 1 nM. All reagents in this experiment were dissolved in ethanol; 0.1% ethanol was used in the control.

100 fM Tam, which slightly increased the number of mononuclear cells). It is notable that this 10 μ M concentration is 10,000-fold higher than that which increased apoptosis of osteoclasts in our model.

Intracellular recording of resting potential

Because both Tam, and to some extent Tor, seemed to influence cell viability, this phenomenon was further studied using UMR-108 cells. We found that resting membrane potentials, when recorded with glass capillary microelectrodes, were in the range of -15 to -40 mV, which is typical for many epithelial cells. The recorded input resistances ranged from 5 to 10 M Ω . The application of Tam resulted in a rapid depolarization of the membrane potential, which stabilized after a few seconds to -10 to -15 mV (Fig. 3B). Application of high K^+ buffer rapidly abolished the membrane potential. In contrast, addition of Tor did not change the resting membrane potential (Fig. 3C). Changing to the high K^+ buffer again abolished the resting potential, which proceeded more rapidly than in the case of Tam. Application of the vehicle (Fig. 3A) or E2 (Fig. 3D) slightly hyperpolarized the resting potential. The input resistance of the cells was clearly reduced both by Tam and Tor application (data not shown), but because of the low initial input resistance of the cells, the exact magnitude of the decrease could not be quantified.

Measurement of cytosolic pH

To study if the effect on membrane potential was accompanied by changes in pH balance, we studied the intracel-

lular pH of the UMR-108 cells. We first measured the pH in a normal buffer, in the presence of physiological concentrations of ions, under which conditions the cells have good opportunities to compensate for abnormal ion fluxes. Under these conditions, the addition of both SERMs caused a significant pH rise, although the effect of Tam on the intracellular pH was much stronger than that of Tor (Fig. 4A). Methanol and E2 had no effect, although, at the beginning of measuring, methanol caused a transient rise of pH. We next tested these compounds in a buffer that did not contain any monovalent cations (Fig. 4B)—cells were first balanced and also loaded in these changed buffers, as described in the Materials and Methods section. We observed no membrane leakage or accompanying cell death over a 20-minute incubation with the SERMs. However, the intracellular pH rose to the level of the external pH in the presence of Tam, whereas Tor, E2, and methanol did not have this effect. In all cases, the pH rise was at least partially compensated by the cells over a longer period of time.

Finally, we used a high K^+ buffer without carbonate and sodium to disable the compensation of possible pH changes. The cells were shown to survive although the membrane potential must have been reversed by high K^+ . Figure 4C shows the pH changes caused by the addition of the compounds investigated. The addition of methanol did not cause any changes in the internal pH, whereas E2 significantly lowered the pH. In the presence of 50 μ M Tam or Tor, the dye freely leaked out of the cells, and this was obviously caused by the disruption of the outer cell membrane of the

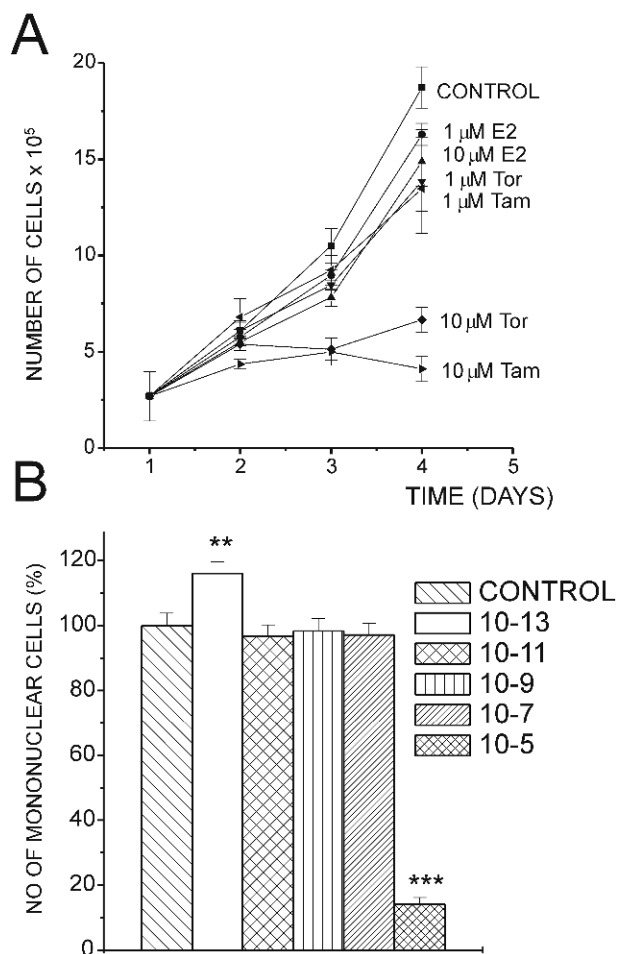


FIG. 2. The effect of E2, Tam, and Tor on the number of nonosteoclast cells. (A) The number of UMR-108 cells was determined by CoulterCounter after treatment with 1 and 10 μ M E2, Tam, and Tor. Each point represents the mean number of cells from three parallel wells \pm SD. (B) The number of mononuclear cells in rat bone marrow cultures. ** p < 0.01, *** p < 0.001. All reagents in this experiment were dissolved in ethanol; 0.1% ethanol was used in the control.

cells. This was followed by the lysis of the cell membrane and the death of the cells. This is demonstrated in Fig. 4D. Similar results were obtained using MCF-7 human breast cancer cells (data not shown).

Proton transport

Dye leakage described above suggested a direct membrane effect of Tam and Tor, and we next measured H⁺ transport in chicken medullary bone-derived microsomal vesicles. Methanol (1%), in which all the compounds were dissolved, was used as a control. Figure 5A shows that 10 μ M Tam and Tor inhibited proton transport by about 80%, whereas E2 had no effect. The addition of E2 did not block the inhibition caused by Tam and Tor (data not shown).

To find out whether inhibition of proton transport was caused by direct inhibition of the V-ATPase or to proton leak through the plasma membrane, we loaded the vesicles with protons by adding ATP only. The compound to be

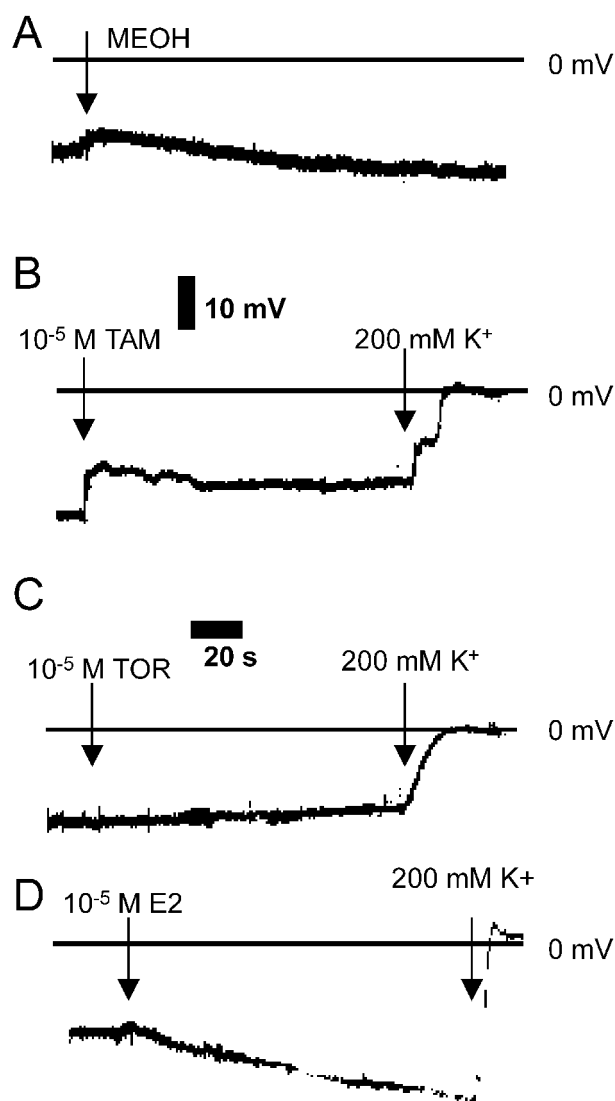


FIG. 3. The effect of (B) Tam, (C) Tor, and (D) E2 on the membrane potential of UMR-108 cells. (A) The control situation (methanol). The data shown represent real-time recordings of membrane potential using intracellular electrodes. High K⁺ was added at the end of the experiment in B–D to show total depolarization. All reagents in this experiment were dissolved in methanol.

tested was added after the formation of the proton gradient. A proton ionophore such as nigericin used as a control compound caused complete leakage of the protons out of the vesicles in 1 minute (data not shown). Both Tam and Tor at 10 μ M concentration had a similar effect, although they were slightly less efficient than nigericin (Fig. 5B). The proton transport at 10 μ M Tam was only 8% of the control. At 5 μ M, it was 24%, and at 1 μ M, it was 79% of the control. The lowest Tam concentration causing detectable proton leakage was 0.5 μ M and the proton transport at this concentration was still 95% of the control. E2 caused only a minor leak (Fig. 5B). Similar results were obtained when membrane vesicles containing gastric H⁺/K⁺ ATPase were tested (data not shown). These results suggested that the

H⁺-transport inhibition by Tam and Tor could be caused by their ability to cause proton leak and not because of the direct inhibition of the proton pump. The compounds either reacted directly with the membrane and formed an ion channel for protons or they bound to an existing membrane channel, opening it for protons. Finally, they possibly interacted with the membrane itself and changed its properties,

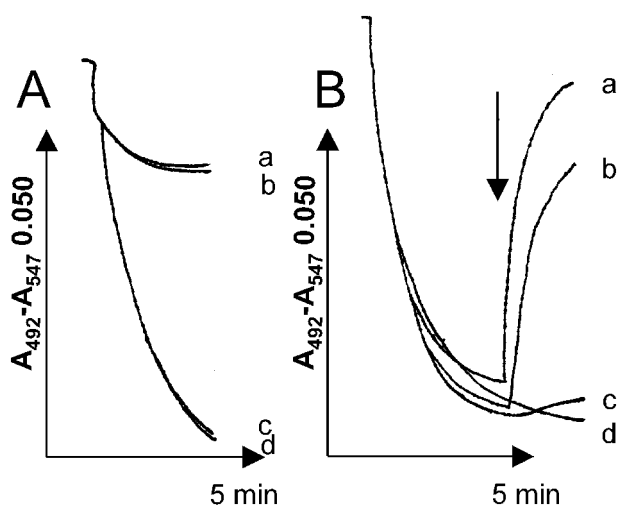
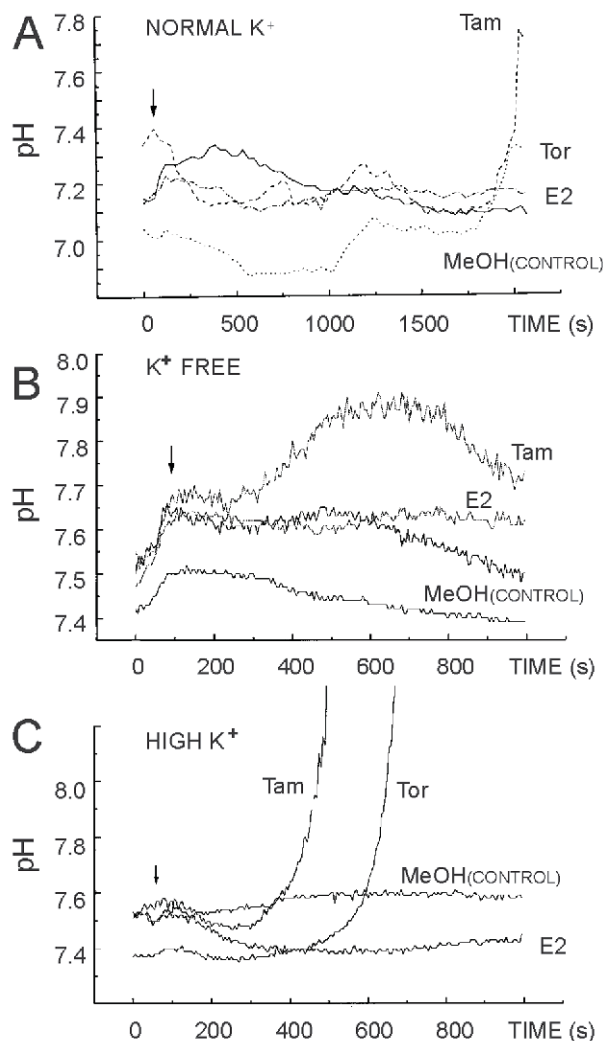


FIG. 5. The effect of E2 and SERMs on H⁺ transport in (A) chicken medullary bone microsomal vesicles and (B) proton leakage from H⁺-loaded chicken medullary bone microsomal vesicles. (A) Each compound was added to the cuvette 5 minutes before measuring for a final concentration of 10 μ M. Curves represent the effects of (a) Tor, (b) Tam, (c) E2, and (d) methanol control. (B) Vesicles were incubated with ATP for 4 minutes, and the tested compound was added to the final concentration of 10 μ M (arrow). Curves in B represent the effects of (a) Tor, (b) Tam, (c) methanol control, and (d) E2.

which may have induced the opening of some voltage-dependent channels.

To test these hypotheses, we studied the effect of added protein-free phospholipid liposomes. As shown in Fig. 6A, the addition of liposomes could block the proton leakage caused by 10 μ M Tam. A 10-fold excess of protein-free membrane fraction compared with plasma membrane vesicles blocked the major part of inhibition from 86% to 5%. Similar results were obtained with 10 μ M Tor, suggesting that these compounds bind directly to the lipid membranes, which leads to the formation of channels allowing proton transition at least. When choline chloride was used instead of K⁺ (or Na⁺) in the assay buffer, Tor lost its ability to cause proton leakage at tested concentrations. Tam acted as effectively in both high and low K⁺ buffers (Fig. 6B).

DISCUSSION

Our data show that the effect of Tam was associated with an increased rate of apoptosis, which obviously led to a

FIG. 4. The effect of E2, Tam, and Tor on the intracellular pH of UMR-108 cells. (D) Six image analyzer pictures of a single UMR-108 cell at different time points when Tam was present at high K⁺ buffer. Numbers presented under each cell picture correspond to time points in C. All compounds were added to samples at 100 s. The measurement time was 16 minutes. The final concentration of each molecule was 50 μ M and the same amount of methanol was present in all groups. (A) Cells were loaded in normal buffer (pH 7.2) and measured in normal buffer (pH 7.8). Intracellular pH was followed for 33 minutes instead of 16 minutes. (C) Cells were loaded in high K⁺ buffer (pH 7.2) and measured in high K⁺ buffer (pH 7.8). (B) Cells were loaded in K⁺-free buffer (pH 7.2) and measured in K⁺-free buffer (pH 7.8).

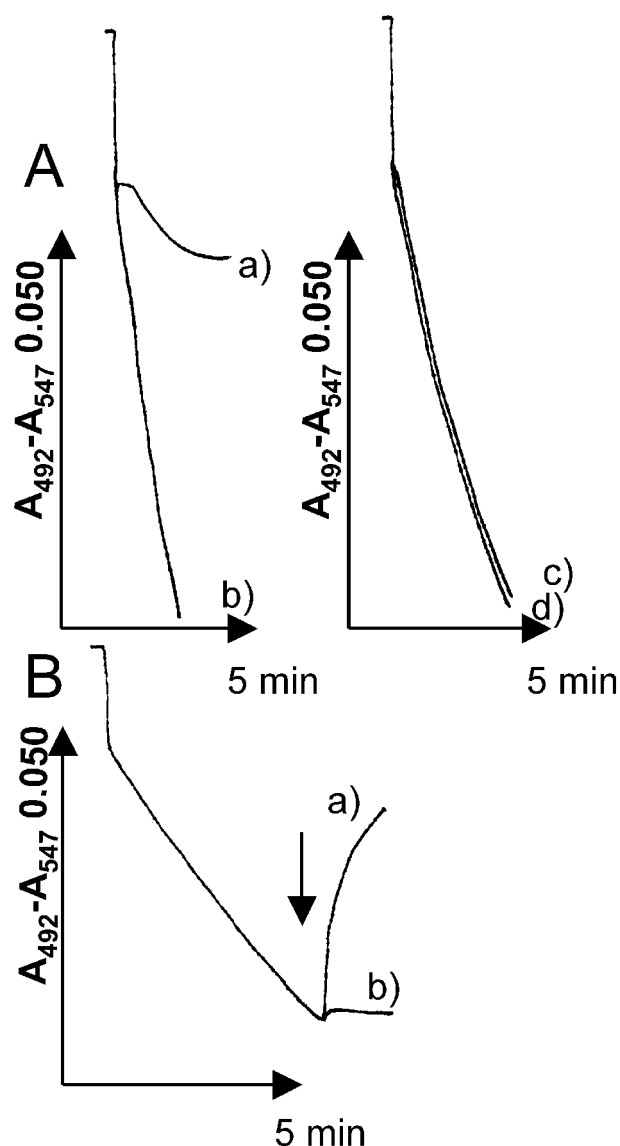


FIG. 6. The effect of phospholipid liposomes on the proton leak induced by (A) Tam and the effect of K^+ removal on the proton transport inhibition by (B) Tam and Tor. In both A and B, the final concentration of antiestrogen was $10 \mu\text{M}$. (A) Curve a represents the effect of Tam on proton accumulation, and curve b represents the methanol control when no phospholipid liposomes were present; curve c represents Tam, and curve d represents methanol control in the presence of phospholipid liposomes. (B) Curve a represents the effect of Tam and curve b represents the effect of Tor in proton leakage in the absence of K^+ .

shortened life span of osteoclasts. In this study, a similar effect could not be demonstrated with E2. In contrast, E2 was able to protect osteoclasts from apoptosis induced by Tam or serum withdrawal. E2 protection against Tam-induced apoptosis may be caused by the specific receptor-mediated mechanisms, but it is also possible that it is caused by E2's independent survival effect, as suggested by E2 opposition of serum withdrawal-induced apoptosis. Our osteoclast data are in line with the previous studies in which Tam, but not E2, affected the viability⁽⁷⁾ or membrane HCL

transport⁽³⁹⁾ of osteoclasts. The viability of osteoblastic cells was affected only in the presence of $10 \mu\text{M}$ or higher concentration of Tam. In contrast, even nanomolar Tam induced apoptosis in osteoclasts, the effect of which may be ER mediated. We suggest that Tam has general cytotoxicity at higher concentrations.

These non-receptor-mediated effects of Tam were further characterized in electrophysiological experiments. In this experimental setting, a depolarization to near zero after the application of high K^+ buffer is to be expected, as the procedure changes the K^+ -equilibrium potential (the Nernst potential) from about -90 mV to about 0 mV . The relatively rapid depolarization reflects the high resting permeability of the plasma membrane to potassium. The Cl^- equilibrium potential in the normal buffer is very likely to be near zero. Typical of cells of epithelial origin,^(36,37) the cell membrane is apparently quite permeable to Cl^- as well, because the resting potential was recorded to be between the potassium and chloride equilibrium potentials. The rapid depolarization caused by application of Tam must have been caused by increased membrane permeability to an ion (or ions) having equilibrium potential positive from the resting membrane voltage. In principle this change could be specific for Cl^- permeability. However, it is more likely that Tam induces a nonspecific increase of permeability that allows leakage of many types of ions through the plasma membrane. In our experiments, the effect of Tam and Tor may be independent on binding to any specific target protein, because the compounds were able to affect protein-free phospholipid liposomes as demonstrated in proton transport experiments.

Our results suggest that Tam and Tor have a detergent-like effect both on isolated membrane vesicles and the membranes of intact cells. However, there was a difference in the response times between proton transport experiments with membrane vesicles and intracellular pH measurements in intact cells. This was likely caused by the compensatory mechanisms by which living cells control the intracellular ion concentrations. Isolated vesicles do not have such a capacity. Depolarization of the plasma membrane obviously decreased the ability of the cells to react to ion fluxes, thus speeding up cell lysis.

The serum concentration of Tam is approximately 0.1 – $1.0 \mu\text{M}$ at normal therapeutic doses.^(40,41) In our experiments, a micromolar concentration of Tam or Tor (1 – $50 \mu\text{M}$) in the absence of serum produced ionophoric effects in vitro in osteoblasts. The addition of serum reduced this effect, which is probably caused by the binding of these SERMs to the protein components of serum and the consequent reduction in the concentration of free, available drug. A considerable effect was, however, still observed in vitro in the presence of 5 – 20% serum. It is thus conceivable that direct membrane effects of Tam and Tor might also play a role in vivo. In addition, the data available show that these compounds accumulate in certain tissues, which can lead to high local concentrations for example in the lung and the liver. In these tissues, up to 60 – 70 times the serum concentration of Tam can be found.⁽²⁹⁾ Thus, it seems likely that Tam and Tor concentrations in vivo can reach the levels required to produce direct membrane effects.

It is not known, however, whether these effects would exist in bone. The local concentrations of Tam or Tor in bone during therapy have not yet been reported. It is likely that osteoblasts and osteocytes are protected, because Tam or Tor has not been reported to cause bone loss. Their effect on resorbing osteoclasts could, however, also involve a receptor-independent component, because the resorption process as such requires an intact and effective ion balancing apparatus.⁽⁴²⁾

Tam and Tor are structurally similar except for one chlorinated ethyl side-chain present in Tor. The difference in the molecular structure has proved, however, important at least for the capacity of the two compounds to generate hepatotoxic metabolites.⁽⁴³⁾ The chlorine atom also makes Tor less polar than Tam, which may make the interaction of Tor with the membranes more difficult. This is in agreement with our results of membrane potential measurements. Depolarization of the membranes in the presence of high potassium concentration enhanced the ionophoric effect of Tor to the level of Tam. According to these results, it can be expected that also in vivo Tor is less potent in inducing ion leakage through the plasma membrane.

Our results differ from some studies^(4,5) in which E2 was reported to induce apoptosis of osteoclasts directly. However, in those studies, the experiments were carried out with purified rabbit osteoclasts⁽⁵⁾ or with murine osteoclasts⁽⁴⁾ in a culture medium containing either 0.1% bovine serum albumin (BSA) or 10% charcoal stripped FCS. Our experiments were conducted in a medium containing non-stripped FCS, and we used nonpurified primary rat bone cells, most of which were nonosteoclasts. It should be noted, however, that although we used a mixed cell population in our cultures, we could still measure different responses on resorption activity⁽³⁾ and apoptosis in osteoclasts.⁽³⁵⁾ We find these conditions satisfactory in modeling the in vivo effects of E2, because the sum effect of E2 always depends not only on its direct effect on osteoclasts but also on the numerous other cells present in the bone tissue.

In conclusion, our results show that Tam, and in some conditions Tor, has an ionophoric effect on the plasma membrane. It is possible that this effect is involved in chemotherapeutic effects associated with the treatment of the patients with these compounds. It is further possible that the distinction between the two SERMs in their ability to affect membranes could at least partly explain the differences in the spectrum of clinical side effects elicited by these drugs. Additionally, we await with great interest the detailed comparative studies between Tam and Tor effects on bone.

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