Regulation of Avian Osteoclastic H^+-ATPase and Bone Resorption by Tamoxifen and Calmodulin Antagonists

EFFECTS INDEPENDENT OF STEROID RECEPTORS^*

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We used highly purified avian osteoclasts and isolated membranes from osteoclasts to study effects of tamoxifen, 4-hydroxytamoxifen, calmodulin antagonists, estrogen, diethylstilbestrol, and the anti-estrogen ICI 182780 on cellular degradation of ^3^H-labeled bone in vitro and on membrane HCl transport. Bone resorption was reversibly inhibited by tamoxifen, 4-hydroxytamoxifen, and trifluoperazine with IC_{50} values of 0.25–1.5 \mu M. Diethylstilbestrol and 17\beta-estradiol had no effects on bone resorption at receptor-saturating concentrations, while ICI 182780 inhibited bone resorption at concentrations greater than 1 \mu M. At these concentrations ICI 182780, like tamoxifen, inhibits calmodulin-stimulated cyclic nucleotide phosphodiesterase activity. Membrane HCl transport, assessed by ATP-dependent acridine orange uptake, was unaffected by 17\beta-estradiol and diethylstilbestrol at concentrations up to 10 \mu M, while ICI 182780 inhibited HCl transport at concentrations greater than 1 \mu M. In contrast HCl transport was inhibited by tamoxifen, 4-hydroxytamoxifen, and the calmodulin antagonists, trifluoperazine and calmidazolium, with IC_{50} values of 0.25–1.5 \mu M. These results suggested the presence of a membrane-associated non-steroid receptor for tamoxifen in osteoclasts. Tamoxifen binding studies demonstrated saturable binding in the osteoclast particulate fraction, but not in the nuclear or cytosolic fractions. Membranes enriched in ruffled border by differential centrifugation following nitrogen cavitation showed binding consistent with one site, K_d = 1 \mu M. Our findings indicate that tamoxifen inhibits osteoclastic HCl transport by binding membrane-associated target(s), probably similar or related to calmodulin antagonist targets. Further, effects of estrogens or highly specific anti-estrogens on bone turnover do not support the hypothesis of a direct effect on osteoclasts by these compounds in this species.

Transport of Ca^{2+} into and out of bone is critical for maintenance of serum calcium activity. This requires continuous bone turnover at variable rates, which is mediated by the osteoclast. However, skeletal mineral is also structurally vital, so osteoclastic activity is regulated by multiple factors, often acting in opposing directions. Several hormonal signals are involved in this regulation, including peptides and low molecular weight factors (1). Steroids including estrogens have major effects on bone turnover (2), but the receptors and intermediary signaling involved are not established. This study was performed to determine the mechanism of steroid-related effects on central biochemical elements of osteoclastic activity.

A limiting biochemical step and the central regulated element of bone turnover is secretion of HCl to dissolve the bone mineral. This is driven by a vacuolar-like H^+-ATPase that is highly expressed in a unique osteoclastic organelle, the ruffled membrane (3). Multiple intermediary cell signals influence the activity of acid secretion, but one of critical interest is intracellular calcium activity and the ubiquitous calcium-binding protein, calmodulin. The unique acid-dependent dissolution of calcium salts produces high local extracellular calcium activity (4), which is reflected in an elaborate osteoclastic calcium regulatory mechanism including a calmodulin-dependent calcium ATPase (5), and factors influencing osteoclastic intracellular calcium activity such as matrix attachment (6). The vacuolar-like H^+-ATPase driving acid secretion in osteoclasts is also calmodulin-dependent, and osteoclasts concentrate calmodulin at the ruffled membrane (7).

The anti-estrogenic compound, tamoxifen, reduces bone turnover (8), suggesting that tamoxifen may be a particularly useful tool to dissect osteoclast control pathways. Tamoxifen, a known calmodulin antagonist (9), is a triphenylethylenedervative with low toxicity and strong antitumor activity, particularly in breast cancer, properties ascribed to its anti-estrogenic activity (10). Tamoxifen may thus regulate osteoclastic activity by either calmodulin or steroid receptor interactions. In contrast to expectations that tamoxifen would cause bone loss because of anti-estrogenic properties (11), it preserves bone mass (8, 12) and has estrogen-like effects on human bone metabolism (13). Tamoxifen has mixed estrogenic and anti-estrogenic effects, which can be tissue-specific. The ethoxyaminoalkyl side chain of tamoxifen is known to be essential for both the anti-estrogenic and calmodulin antagonistic effects (14). The estrogen receptor is a calmodulin-binding protein (15), and derivatives of estrogen substituted with the ethoxyaminoalkyl side chain of tamoxifen prevent the binding of calmodulin to the estrogen receptor (16).

Consequently, we studied the effects of tamoxifen and its active metabolite, 4-hydroxytamoxifen, on osteoclastic HCl transport and cellular activity, and compared these effects to those of calmodulin antagonists, estrogens, and a specific anti-estrogen. We report that tamoxifen and 4-hydroxytamoxifen inhibit membrane acid transport and osteoclastic bone resorp-

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tion with dose responses similar to the calmodulin antagonists, while neither estrogens nor a highly specific steroid-estrogen showed measurable effects at relevant concentrations. In keeping with these findings, tamoxifen binds to osteoclast cell membrane fractions with a dissociation constant consistent with concentrations observed to have functional effects on whole cell activity and cell membrane ATP-dependent HCl transport. These data support a model for control of osteoclastic acid secretion by tamoxifen and related compounds that is not directly related to estrogen receptors, and further suggest that compounds such as tamoxifen act on the acid-secreting membrane by a mechanism similar to other calmodulin antagonists.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures—**Osteoclast-rich cell fractions were obtained from medullary bone of laying hens, Gallus domesticus. Animals on a Ca2+-restricted diet produced large numbers of osteoclasts, ~50% of the medullary bone cell mass, which were harvested and enriched by sieving through 110-μm nylon. Erythrocytes were lysed in hypotonic media and cells sedimented through 70% serum to recover the dense osteoclasts as described (17). Typically 85–90% of cell nuclei at this stage are in osteoclasts as assessed by tartrate-resistant acid phosphatase activity. Unless noted, assays reported herein used osteoclasts further purified by binding to nitrocellulose (18). Osteoclast-enriched fractions plated at 2 × 10^5/cm^2 with 60 μg/cm^2 of 20-40-μm (unlabeled) bone fragments for 36 h to allow viable osteoclasts to attach to the bone. Plates were washed gently to eliminate unattached cells, and bone fragments with attached cells are then dislodged by vigorous washing with ice-cold phosphate-buffered saline. Bone was then recovered, with attached osteoclasts, by sedimentation twice for 5 min in 10-cm columns of phosphate-buffered saline. Bone-attached cells were characterized by tartrate-resistant acid phosphatase staining and using a monoclonal antibody, 121F, reacting specifically with avian osteoclasts (Fig. 1), kindly supplied by Philip Osdoby, Washington University, Saint Louis, MO (18). Cells were fixed 2 h in 1% phosphate-buffered formalin at 4°C and incubated 30 min at 20°C with 10 μg/ml streptomycin, 100 μl/10 ml dilute 1:100 or an equivalent dilution of nonimmune ascites in phosphate-buffered saline with 0.05% polyoxyethylene sorbitan monoleate and 1% bovine serum albumin, washed, and incubated with fluoresceinated goat anti-mouse antibody (Sigma) at 1:500 dilution to identify bound primary antibody, washed, and examined by epifluorescence using 450–490 nm excitation and a 520-mm barrier filter. Tartrate-resistant acid phosphatase was demonstrated using naphthol AS-D-chloroacetate and nitroblue tetrazolium solution as substrate and fast garnet G-4 (2-methylphospho-3-azobenzene diazonium hydrochloride to show the product as red color, in the presence of 4 mM tartrate at pH 5.6. Cells were incubated in Dulbecco’s modified Eagle’s medium without phenol red (to preclude artificial steroid-like dye effects) at 37°C in humidified air with 5% CO2. Media contained 100 μg/ml streptomycin, 100 μl/ml penicillin, and 10% serum (5% chicken, 5% newborn calf); endogenous serum steroid hormones were eliminated by stirring with activated charcoal.

**Bone Resorption—**Bone degradation by avian osteoclasts was quantified by radiometric assay using rat bone labeled in vivo with [2,3,4,5-3H]labeled bone in methyl as described (17). This assay directly measures dissolution of the bone, insoluble component of bone, reflecting complete hydrolysis of mineral and non-mineral components, and is essentially unaffected by physicochemical changes. (17). Characterization of degradation products and comparison with assay results, using [4,5Ca]-labeled bone or cell-pitting as reported (17, 19), indicate that this assay is specific for osteoclasts with intact osteoclasts and histologic background and interassay variability. In this application, 100–200 μg of 20–40-μm bone fragments at 25 dpμg of [3H]labeled bone were added to 2–3 × 10^5 osteoclasts/cm^2 tissue culture well. [3H]Proline released from the labeled bone into the medium was measured relative to no-cell controls, using periods less than 5 days, where activity is essentially linear. The substrate was hydrolyzed with 6% HCl at 60°C, 18 h, for determination of specific activity by scintillation counting.

**Membrane Vesicle Preparation and HCl Transport Assay—**Cells were fragmented by nitrogen cavitation (20) using 10^5 cells in 20 ml of 250 mM sucrose, 20 mM KCl, 0.5 mM EDTA, 1 mM dithiorethiol, 10 mM Tris, pH 7.0, with explosive decompression following 30 min at 35 atm N2 (1,6 megascalps), 4°C. Sequential centrifugation at 4°C (1,000 × g, 5 min; 9,000 × g, 10 min) removed cell fragments, nuclei, and mitochondria, and the vesicular fraction was obtained by centrifugation of the 5,500 × g supernatant at 37,000 × g for 1 h. Cell and vesicle labeling showing that ~50% of membranes in these preparations are ruffled border, and cytoplasmic enzymes are ~1% of whole cell levels is described (20). To assay Mg2+-ATP-dependent HCl transport in response to the test compounds, membrane vesicles were suspended in 120 mM KCl, 20 mM NaCl, 10 mM HEPES, pH 7.4, at 1.5 mg/ml protein and incubated (30 min, 4°C) to allow vesicles to stabilize. Acid trans- port vesicles was determined by monitoring acridine orange uptake by fluorescence spectrophotometry with excitation at 468 nm and digital recording of averaged emission at 540 nm (E_540) at 5-s intervals, using ~25 μg of vesicle protein (15 μl of reconstituted vesicles) in 2.5 ml of 1 mM ATP, 3.3 μM acridine orange, 120 mM KCl, 20 mM NaCl, 10 mM HEPES, pH 7.4, at 37°C in a stirred cuvette. Transport was initiated with 2 mM Mg2+ ATP transport was initiated with 2 mM Mg2+ and was stopped in fluorescence on addition of a 300-fold molar excess of NH4Cl over acridine orange to replace acridine accumulated in acid compartments with the non-fluorescent weak base. Differences were determined 15 s after NH4Cl addition to eliminate mixing artifacts. Vesicle activity was stable >10% at 4°C from 30 min to 5 h after reconstitution, allowing several comparisons with each vesicle preparation. Antibody labeling showing that osteoclasts are the predominant source of H+ ATPase in medullary bone has been reported (3); control assays using membrane vesicles from other bone cells, including narrow monocytes isolated as described (21), showed negligible activity.

**Tamoxifen Binding—**Osteoclasts were cultured in six-well plates as described above. Cells were washed with phosphate-buffered saline at 4°C, scraped into 4 ml of 0.5 M Tris, 250 mM sucrose, 1 mM EGTA, 1 mM KH2PO4, 1 mM dithiothreitol, pH 7.0, and homogenized by a Teflon pestle at 2,600 rpm. Cytosolic, crude nuclear and particulate fractions and were prepared as follows; the homogenate was centrifuged at 1,000 × g for 5 min (the pellet represents the crude nuclear fraction), and the supernatant centrifuged 40,000 × g for 30 min. The high-speed pellet (particulate fraction) was resuspended in 3 ml of homogenization buffer, while the corresponding supernatant represents the cytosolic fraction. Separate experiments utilized membrane vesicles enriched for ruffled membrane, which were isolated by the nitrogen cavitation procedure described in the previous section. Tamoxifen binding was determined by incubation with 0.63 nm (ring-[3H]tamoxifen, 83 Ci/mmol; American Radiolabeled Corp.) with increasing concentrations of unlabeled tamoxifen (10 nM to 30 μM). Reactions were incubated 30 min at 30°C and stopped by addition one volume of 0.2% bovine γ-globulin with incubation on ice. One volume of 5% polyethylene glycol (M, 8,000), 0.1 Na2HPO4, pH 7.5, was added, samples were vortexed and placed on ice 15 min. Bound label was pelleted (30,000 × g, 15 min) supernatant aspirated. Pellets were washed twice with 1 ml of 12.5% polyethylene glycol. Pellet radioactivities were determined by liquid scintillation counting. Protein concentrations were assayed (Bradford DC, Bio-Rad), and binding was calculated as tamoxifen bound/mg of cell protein.

**Phosphodiesterase Assay—**Activity of calmodulin stimulated cyclic nucleotide phosphodiesterase was measured as the decrease in fluorescence of 5 μM of cyclic GMP derivative, 2-(N-methyl-3-aminopropyl)-5′-cytosine-3′-5′-cyclic monophosphate (Molecular Probes, Eugene, OR) (22), during a 10-min assay at 37°C, as described (23). Fluorescence at 540 nm was measured with 280 nm excitation using an Aminco-Bowman (Urbana, IL) Series 2 luminescence spectrophotometer. Assays contained 10 mM MOPS, pH 6.8, 90 mM KC1, 5 mM MgCl2, 1 mM EGTA, 1 mM CaC12, (25 μM free Ca2+), 8 μM unlabeled cyclic GMP, 4 μM cyclic nucleotide phosphodiesterase (gift of R. Kincaid, National Institutes of Health, Bethesda, MD), and 15 μM calmodulin (Ocean Biologies, Edmonds, WA). Background activity in the absence of calmodulin was subtracted to calculate the calmodulin-stimulated activity. Tamoxifen or 1C182780 was preincubated 5 min with the enzyme prior to calmodulin addition.

**Statistical Methods—**Results are means of quadruplicate determinations unless noted. Error bars indicate standard error of the mean. Groups were compared by analysis of variance or paired Student’s t test; differences are concluded if the null hypothesis is rejected at 5% confidence.

**RESULTS**

**Stemid and Calmodulin Effects on Avian Osteoclast Activity—**Because variable findings of steroid effects on bone are reported (3, 24), the cell preparations used were affinity-purified and the composition of the resultant cell preparations was characterized using the monoclonal antibody 121F specific for

1 The abbreviation used is: MOPS, 4-morpholinopropanesulfonic acid.
avian osteoclasts (18) and by tartrate-resistant acid phosphatase activity. Essentially all of the material in these preparations stains with the osteoclast-specific antibody (Fig. 1), except for non-cellular bone fragments used in the isolation, to which some cells remain attached (Fig. 1A, arrows, transmitted light). The antibody to some extent outlines the cells, suggesting membrane-associated antigen (Fig. 1B, epifluorescence) as reported (18). Similarly, essentially all purified cells are tartrate-resistant acid phosphatase-positive (Fig. 1C), with only acellular bone non-reactive. Non-immune serum controls for 121F antibody staining were negative (Fig. 1D). Since essentially all cells in these preparations are osteoclasts, the effects on other steroid-receptor containing bone cells that may mediate secondary effects on osteoclasts, such as osteoblasts, are practically eliminated, allowing direct comparisons of the test compounds on osteoclastic bone degradation.

17-β-Estradiol and diethylstilbestrol did not inhibit resorption of metabolically labeled bone by affinity-purified avian osteoclasts at concentrations meaningful with respect to receptor-mediated effects (Fig. 2). Estradiol had no measurable effect on osteoclastic bone resorption (Fig. 2A). A trend toward increased activity, on the order of 10%, was seen with diethylstilbestrol at concentrations greater than 10⁻¹² M (Fig. 2B), but was not statistically different. Affinity constants of estrogen receptors for these ligands are on the order of 10⁻¹⁰ M, so that concentrations of 10⁻⁸ M, and certainly 10⁻⁷ M, would be saturating even if the 10% serum proteins in the assay medium reduced the effective free steroid activity by an order of magnitude. Similarly, the specific anti-estrogen ICI 182780 had no effect on activity of affinity-purified avian osteoclasts at physiologically meaningful concentrations (Fig. 2C). ICI 182780 inhibited bone resorption only at concentrations greater than 10⁻⁶ M, with 50% inhibition at approximately 10⁻⁵ M. The inhibition was reversible on removal of the compound, and so is not related to cell death. Estradiol, diethylstilbestrol and anti-estrogen effects on degradation of [³H]proline-labeled bone were also tested in partially purified osteoclast preparations, made by serum sedimentation of cells extracted from medullary bone of calcium-deprived laying hens but without bone affinity purification. These results were qualitatively similar to the assays performed with affinity-purified osteoclasts.

Tamoxifen, 4-hydroxytamoxifen, and the calmodulin antagonist trifluoperazine inhibited bone resorption by purified osteoclasts at physiologically relevant concentrations (Fig. 3), in

![Fig. 1. Characterization of osteoclast preparations.](image-url) Osteoclasts were isolated by bone affinity binding (see "Experimental Procedures") and characterized by 121F monoclonal antibody and tartrate-resistant acid phosphatase activity (20, 21). Scale markers indicate 15 μm. A, a field of cells is shown by transmitted light; bone fragments used in the isolation are seen as refractile angular acellular material (arrows). B, fluorescent-labeled 121F antibody tags all of the cells in the same field as in panel A. Some cells show a rim of bright stain suggesting membrane-associated reactivity (arrow). C, tartrate-resistant acid phosphatase was demonstrated using napthol 6-bromo-2-phospho-3-naphthyl-2-methoxyanilide phosphate as substrate, in 4 mM tartrate at pH 5.6, and fast garnet 2-methyl-4-[(2-methylphenyl)-azo]benzene diazonium hydrochloride to show the product as red color. The affinity isolated cells are all positive, while bone fragments used in the procedure do not react (arrows). D, control cells similar to those in panel B, but reacted with non-immune serum at the same concentration.
contrast to the estrogen-related compounds which were ineffective at receptor-saturating concentrations (Fig. 2). Tamoxifen, 4-hydroxytamoxifen and trifluoperazine inhibited bone resorption in a concentration-dependent manner with maximal inhibitions near 7 μM. Effects were saturating and reversible: Half maximal inhibition was 1 μM for all compounds. Removal of the substances resulted in return of osteoclastic bone resorption to control levels during an additional 3-day incubation (data not shown), indicating that these concentrations did not kill the osteoclasts. While the effective concentrations of these compounds are 10²- to 10³-fold greater than those relevant to steroid receptors, these IC₅₀ values are typical for calmodulin-antagonist effects and are similar to peak serum levels obtained for tamoxifen in the treatment of breast cancer, 0.5-1 μM. As with estrogen and anti-estrogen experiments, similar results were obtained when partially purified osteoclasts were used.

Because ICI 182780 inhibited osteoclasts at concentrations above 1 μM where the calmodulin antagonists were also effective, we tested whether this anti-estrogen is also a calmodulin antagonist. This would not be inconsistent with the specificity of ICI 182780 anti-estrogenic effects (27) because in that capacity its Kᵦ is 10²- to 10³-fold lower. Further, a variety of compounds with hydrophobic planar groups and flexible polar side chains, a description that fits ICI 182780, are calmodulin antagonists. To test calmodulin inhibition without introducing systematic bias, we used an unrelated in vitro calmodulin-stimulated phosphodiesterase assay. Both tamoxifen and ICI 182780 inhibited this calmodulin-dependent system similarly.
with half-maximal effects at \(-2-4 \mu M\) (Fig. 4).

Combined Effects of Tamoxifen and Trifluoperazine—The similarity of tamoxifen and trifluoperazine action was tested by determining whether combination of the compounds at submaximal inhibitory concentrations would be complementary (Fig. 5A). Bone resorption was inhibited by 0.7 \(\mu M\) tamoxifen or trifluoperazine alone (columns 2 and 3), and in the presence of 0.7 \(\mu M\) of both tamoxifen and trifluoperazine was inhibited further, to a level approaching maximal inhibition (column 4). Thus, submaximal inhibitory concentrations of these two compounds were additive. Addition of estrogen (1 \(\mu M\)) had no effect over 0.7 \(\mu M\) of either trifluoperazine or tamoxifen (not illustrated). However, maximal inhibitory concentrations of tamoxifen or trifluoperazine were not additive, as demonstrated by the combination of 7 \(\mu M\) tamoxifen and 3 \(\mu M\) trifluoperazine (Fig. 5B, column 6), which is not different from the maximal concentrations of either agent alone.

Inhibition of ATP-dependent Membrane HCl Transport—Osteoclastic HCl transport is central to bone degradation (3) and calmodulin-dependent (7). The activity of tamoxifen or trifluoperazine on bone degradation suggested that these compounds may directly inhibit osteoclastic HCl transport. In contrast, since estrogen did not effect bone degradation, it would not be expected to affect osteoclastic acid secretion. We compared the effects of each compound on membrane ATP-dependent acid transport.

Vesicle acidification was not affected by 1 \(\mu M\) 17-\(\beta\)-estradiol (Fig. 6A), and neither diethylstilbestrol nor ICI 182780 had measurable effects at meaningful concentrations relative to steroid receptors (10\(^{-8}\) M). Tamoxifen and the calmodulin antagonist trifluoperazine completely inhibited vesicle acidification at concentrations consistent with their effects on cellular activity (Fig. 6, B and C, respectively). Concentration dependence of inhibition of ATP-dependent membrane acid transport by tamoxifen, trifluoperazine, and another calmodulin antagonist, calmidazolium, are summarized in Fig. 7. Half-maximal inhibitory concentrations were 0.25, 1.5, and 1.0 \(\mu M\) for tamoxifen, trifluoperazine, and calmidazolium, respectively. The half-maximal inhibitory concentrations of tamoxifen and trifluoperazine on vesicle acidification were similar to their IC\(_{50}\) values on bone resorption (Fig. 3). Membrane vesicle acidification was inhibited by 4-hydroxytamoxifen, the major metabolite of tamoxifen, similarly to tamoxifen. ICI 182780 inhibited vesicle acidification at 10\(^{-5}\) M, in keeping with effects observed in the bone resorption experiments and with its inhibition of calmodulin-dependent phosphodiesterase activity at this concentration.

Tamoxifen Binding to Osteoclast Membrane Fractions—Tamoxifen had effects on membrane transport, but is also known to bind proteins including estrogen receptors that are found in the cytosol and nucleus. We measured tamoxifen binding as a function of concentration in particulate (membrane), cytosolic, and nuclear fractions of affinity-purified osteoclasts to determine whether tamoxifen binding sites were present in each fraction. Tamoxifen binding saturated at \(-3 \mu M\) in the membrane fraction. Binding in cytosol and nuclear fractions was 2-7-fold/mg of protein lower than the membrane fraction, and non-saturable, indicating nonspecific binding (Fig. 8). Binding experiments were repeated using a preparation enriched in the acid-transporting membrane obtained by nitrogen cavitation. In these preparations, tamoxifen binding saturated at \(-2 \mu M\) (Fig. 9A), and Scatchard analysis demonstrated a single binding affinity with an apparent K\(_D\) of 1 \(\mu M\) (Fig. 9B) and \(5 \times 10^6\) sites/cell.
Regulation of Osteoclastic HCl Transport by Tamoxifen

**FIG. 6. Effect of 17-β-estradiol, tamoxifen, and trifluoperazine on osteoclast membrane vesicle acidification.** ATP-dependent acid uptake was measured in avian osteoclast membranes isolated by nitrogen cavitation and differential centrifugation. Acid transport was monitored by measuring the decrease in fluorescence at 540 nm with excitation at 468 nm of acidine orange due to uptake into vesicles (vertical axis) as a function of time (horizontal axis). Assays were performed with vehicle alone (closed symbols) or with vehicle plus test compounds (open symbols) 1 μM 17-β-estradiol (A), 2 μM tamoxifen (B), and 10 μM trifluoperazine (C). Test compounds were added to the assay mixture 5 min prior to addition of 2 mM MgCl₂. Control acidification was confirmed by fluorescence recovery on washout of the fluorescent weak base with 1 mM NH₄Cl (closed arrow, right).

**DISCUSSION**

Estrogens are bone sparing agents, but their mechanism of action is not clear. Which bone cells have estrogen receptors is controversial. Further, whether estrogenic effects are mechanistically related to the effects of bone-sparing compounds such as tamoxifen is enigmatic. It has been proposed that tamoxifen inhibits osteoclast activity by antagonizing estradiol binding (8, 26). However, other mechanisms might lead to similar findings. For example, the calmodulin antagonist trifluoperazine inhibits breast cancer cell growth similarly to tamoxifen, but independently of the estrogen receptor (27), and tamoxifen is both an anti-estrogen and a calmodulin antagonist. We studied effects of estrogen, tamoxifen, and related compounds on osteoclastic bone resorption and a key regulated process in bone resorption, membrane HCl transport, to resolve these points.

Bone affinity-purified avian osteoclasts were used to limit observations to direct osteoclast interactions (Fig. 1). This technique uses a small quantity of fragmented bone with a large surface area to concentrate osteoclasts, which attach to bone; other cells attach nonspecifically according to the substrate where they settle, which is 99% cell culture plastic (using other cells attach nonspecifically according to the substrate where they settle, which is 99% cell culture plastic (using 10-cm tissue culture plates and 2 mg of 20–40-μm bone). Thus, the ratio of osteoclasts to contaminants in the bone-attached fraction is, under ideal conditions, improved by 2 orders of magnitude, and non-osteoclastic effects, such as signals that may be generated from steroid receptors in other bone cells, are eliminated. This simplifies interpretation of results, and permits more detailed biochemical dissection of the bone resorption process.

We find that the estrogens 17-β-estradiol and diethylstilbestrol, as well as the highly specific steroidal anti-estrogen ICI 182780, have no effect on osteoclastic activity at receptor-saturating concentrations (Fig. 2). At extremely high concentrations, over 10⁻⁶ M, effects were variable; cell activity in the presence of diethylstilbestrol was slightly increased, although the difference was not statistically different. On the other hand, ICI 182780 inhibited bone resorption at concentrations over 10⁻⁶ M. ICI 182780 binds the estrogen receptor with an
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Fig. 9. Tamoxifen binding to osteoclast membranes containing a high proportion of HCl transporting membrane. Aliquots of membrane fractions produced by nitrogen cavitation (see “Experimental Procedures”) were incubated with 0.63 nm [3H]tamoxifen and 0–30 μM unlabeled tamoxifen. Samples were precipitated, washed in polyethylene glycol, and counted to determine bound and free tamoxifen. Binding at 30 μM unlabeled tamoxifen was subtracted as nonspecific binding. A, tamoxifen binding in ruffled membrane-enriched preparations. Means of duplicate determinations are shown; these data are typical of results from three separate experiments. B, Scatchard analysis of binding data for ruffled membrane-enriched preparations. The apparent \( K_d \) is 1 μM, with 5 × 10^6 sites/cell. This result suggests that tamoxifen at micromolar concentrations binds an abundant membrane-associated protein in osteoclasts.

The apparent \( K_d \) of 10^-9 M (25), and physiological estrogen concentrations are 10^-10 to 10^-9 M. In postmenopausal women, plasma 17-β-estradiol is ~1.5 × 10^-10 M, while premenopausal levels are ~7.4 × 10^-10 M (28), and receptor affinities are in this range. Thus, these effects of ICI 182780 are observed at 1000-fold or greater than their receptor \( K_d \) values, and do not represent estrogen receptor-mediated effects. Estrogen and diethylstilbestrol did not effect bone resorption at all concentrations tested (10^-14 to 10^-5 M).

There are numerous reports of effects of estrogen on bone mass not directly attributable to osteoclastic estrogen-receptor binding (2, 8, 29–32). Our findings are not inconsistent with these results, which point to effects on osteoblasts (29), membrane-associated estrogen binding different from the classical estrogen receptor and of uncertain significance (30), or to effects on cell number or activity related to cell differentiation (8, 31, 32). None of these processes are modeled in our system, which specifically examined purified osteoclasts and osteoclast membrane HCl transport.

On the other hand, it has been reported that 17-β-estradiol directly inhibits avian osteoclastic bone resorption, almost completely, with half-maximal effects at ~10^-11 M (24). Our results are not consistent with this study. Because of this controversial report, our cell preparations were carefully characterized (Fig. 1) and results were repeated with multiple cell preparations. In addition to estradiol, the general estrogen agonist diethylstilbestrol and the highly specific anti-estrogen ICI 182780 were tested. None of these compounds affected bone resorption at or below 10^-6 M. Assays measuring pit formation by avian and rat osteoclasts were run in an attempt to demonstrate an estradiol effect at submicromolar levels; differences from controls were not seen. We conclude that the results reported by Oursler et al. (24) are not reproduced under the conditions used here, which included essentially homogeneous preparations of osteoclasts, phenol-red free medium and charcoal-stripped sera to avoid possible steroid-like effects of the phenol red and effects of serum steroids. The report of estradiol effects in avian osteoclast cultures (24) may thus reflect effects of non-osteoclastic cells, in vitro differentiation, or medium components not present at measurable levels in our system.

In contrast, we found that the triphenylethylene compound tamoxifen had clear inhibitory effects on osteoclastic bone resorbing activity (Fig. 3A). 4-Hydroxytamoxifen (the principal tamoxifen metabolite) and the calmodulin antagonist trifluoperazine had similar effects (Fig. 3, B and C, respectively). In addition, the effects of tamoxifen and trifluoperazine were additive at submaximal inhibitory concentrations (Fig. 5A). Additivity was not observed when one compound was present at maximal inhibitory concentration (Fig. 5B). These results suggest that the inhibitory effect of tamoxifen is related to a calmodulin-dependent signaling mechanism. Tamoxifen and trifluoperazine inhibit calmodulin-dependent cyclic nucleotide phosphodiesterase activity with IC_{50} values of 1–3 μM, supporting this hypothesis. On the other hand, tamoxifen binds both estrogen (10) and anti-estrogen (33) receptors, and these properties are believed to be the basis of its beneficial effects in malignancies such as breast cancer. However, the lack of observed estrogenic or anti-estrogenic effects on osteoclastic activity at receptor-saturating concentrations indicate that neither of these mechanisms is responsible for the observed osteoclastic effects. The calmodulin antagonist activity of tamoxifen (9, 34) depends on its ethoxyaminoalkyl side chain, which is also essential for its anti-estrogenic effects (16). Earlier work on osteoclastic activity points to an important role for calmodulin interactions in acid secreting activity, including a high concentration of calmodulin at the acid secreting ruffled membrane and calmodulin antagonist inhibition of osteoclast membrane acid transport (7).

We investigated this hypothesis further by comparing the effects of tamoxifen and the calmodulin antagonists trifluoperazine and calmidazolium on ATP-dependent membrane acid transport (Figs. 6–7). Tamoxifen, trifluoperazine, and the highly specific calmodulin antagonist calmidazolium were all potent inhibitors of HCl transport. Estrogen and diethylstilbestrol had no effect. The anti-estrogen ICI 182780 inhibited vesicle acidification at concentrations over 1 μM, as did the calmodulin antagonists. However, it is also a calmodulin antagonist at these concentrations (Fig. 4). ICI 182780, tamoxifen, and other calmodulin antagonists all act consistently at low micromolar concentrations by inhibiting bone resorption, osteoclast membrane vesicle acidification, and phosphodiesterase activity. This argues for a related inhibitory mechanism for these compounds, a disruption of calmodulin-dependent signaling. However, calmodulin-dependent control mechanisms are very complex and involve a large number of specific calmodulin-protein interactions with different properties. For example, the half-maximal inhibitory concentration of tamoxifen differed by severalfold in the membrane transport assay (Fig. 6) and in the phosphodiesterase assay (Fig. 4); this is likely due to differences in the particular calmodulin-protein interactions present in the different assay procedures.

2 J. P. Williams, H. C. Blair, and J. M. McDonald, unpublished observations.
A potential effect with membrane transport experiments such as those shown in Fig. 6 is that the antagonists tested are themselves weak bases, which could affect acridine orange distribution. Accumulation of weak bases in acid compartments depends mainly on concentration, membrane permeability of the free base, and pKₐ; high molecular weight alkylamines such as tamoxifen typically have pKₐ ~11 and their membrane-permeable uncharged forms are present at concentrations too low, at pH 7.4 (the assay buffer pH), to compete effectively with acridine orange (pKₐ 9.4). Further, effective concentrations of the antagonists were 0.25-1 μM (Fig. 7). 8-30% of the acridine concentrations, suggesting that such artifacts would be much smaller than the observed effects even if pKₐ were near that of acridine orange. However, to rule out such effects, control assays were run with 1 μM NH₄Cl included (pKₐ 9.3, competes with 3.3 μM acridine at 300 μM; see “Experimental Procedures”). This reduced acridine orange quenching less than 5%, indicating that the effects of the compounds tested cannot be due to nonspecific effects of their amine groups on the assay.

The results of the membrane transport assays suggested that a tamoxifen binding site is present in osteoclast membranes. Tamoxifen binding was saturable in crude membrane fractions (Fig. 8). In contrast, tamoxifen binding in the nuclear and cytosolic fractions was non-saturable (Fig. 8), indicating nonspecific binding. Because ATP-dependent vesicle acid uptake was directly inhibited, a simple binding to the acid-secreting membrane was hypothesized. Ruffled border-rich cell membranes produced by nitrogen cavitation and differential centrifugation demonstrated saturable high affinity membrane binding with a single apparent Kᵦ of 1 μM (Fig. 9). This result is obtained under conditions that may vary substantially from those in living cells in terms of calcium activity, buffer composition, and other variables. Despite these limitations, binding of tamoxifen at low micromolar concentration to this osteoclast fraction enriched in acid-translocating membrane likely reflects a key molecular interaction of the inhibitor with an osteoclastic protein. There were 5 × 10⁶ binding sites/cell, consistent with an abundant membrane-associated protein, but not a steroid receptor. It is likely that this represents interaction with membrane-bound calmodulin or calmodulin-binding proteins.

Our observations indicate that tamoxifen directly inhibits osteoclast membrane acid transport by a mechanism independent of cytosolic steroid receptors. Further, our results show that critical elements in osteoclastic acid secretion are similarly affected by tamoxifen, calmodulin antagonists, and, at high concentrations, ICI 182780. Whether the effects of calmodulin on osteoclast acid secretion derive from a direct effect of calmodulin on the H⁺-ATPase, its charge-coupled Cl⁻ conductance, or are mediated secondarily by calmodulin-binding proteins is unknown. Further, identification of specific protein interactions of tamoxifen will be required to determine whether the similarity of pharmacological effects of tamoxifen and calmodulin antagonists reflect interactions with the same or related proteins.

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