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Differential Effects of Tamoxifen-Like Compounds on Osteoclastic Bone Degradation, H^+-ATPase Activity, Calmodulin-Dependent Cyclic Nucleotide Phosphodiesterase Activity, and Calmodulin Binding

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Abstract We studied effects of calmodulin antagonists on osteoclastic activity and calmodulin-dependent HCl transport. The results were compared to effects on the calmodulin-dependent phosphodiesterase and antagonist-calmodulin binding affinity. Avian osteoclast degradation of labeled bone was inhibited ~40% by trifluoperazine or tamoxifen with half-maximal effects at 1–3 μM. Four benzopyrans structurally resembling tamoxifen were compared: *d*-centchroman inhibited resorption 30%, with half-maximal effect at ~100 nM, cischroman and CDRI 85/287 gave 15–20% inhibition, and *l*-centchroman was ineffective. No benzopyran inhibited cell attachment or protein synthesis below 10 μM. However, ATP-dependent membrane vesicle acridine transport showed that H^+-ATPase activity was abolished by all compounds with 50% effects at 0.25–1 μM. All compounds also inhibited calmodulin-dependent cyclic nucleotide phosphodiesterase at micromolar calcium. Relative potency varied with assay type, but *d*- and *l*-centchroman, surprisingly, inhibited both H^+-ATPase and phosphodiesterase activity at similar concentrations. However, *d*- and *l*-centchroman effects in either assay diverged at nanomolar calcium. Of benzopyrans tested, only the *d*-centchroman effects were calcium-dependent. Interaction of compounds with calmodulin at similar concentrations were confirmed by displacement of labeled calmodulin from immobilized trifluoperazine. Thus, the compounds tested all interact with calmodulin directly to varying degrees, and the observed osteoclast inhibition is consistent with calmodulin-mediated effects. However, calmodulin antagonist activity varies between specific reactions, and free calcium regulates specificity of some interactions. Effects on whole cells probably also reflect other properties, including transport into cells. J. Cell. Biochem. 66:358–369, 1997.

Key words: calmodulin antagonists; calmodulin binding proteins; osteoclast; phosphodiesterase; H^+-ATPase; trifluoperazine; centchroman; cischroman; calcium; bone resorption

Abbreviations: acridine orange, 3,6-bis (dimethylamino) acridine; CDRI-85/287, 2-[4-(2-N-piperidinoethoxy) phenyl]-3-phenyl [1,4-H] benzopyran; centchroman, (*d,l*)-2-dimethyl-*trans*-[3-phenyl, 4-(2-N-pyrolidinylethoxy) phenyl]-7-methoxy-1-chroman; cischroman, (*d,l*)-2-dimethyl-*cis*-[3-phenyl, 4-(2-N-pyrolidinylethoxy) phenyl]-7-methoxy-1-chroman; PBS, phosphate-buffered saline; SEM, standard error of the mean; tamoxifen, 2[4 (*trans*-1,2-diphenyl-2-butenyl)phenoxy]-N,N-dimethylethaneamine; trifluoperazine, 2-trifluoromethyl-10-[3′-(4-methyl-1-piperazinyl) propyl] phenothiazine.

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Bone turnover is a continuous metabolic process that maintains skeletal strength, and the major component of bone is a basic calcium salt that is the calcium reserve in vertebrates. Thus, bone turnover is also central to maintenance of serum ionized calcium levels. Osteoclasts are specialized giant cells that dissolve bone mineral by pumping HCl across a specialized organelle, the ruffled membrane, into an extracellular compartment between the cell and bone. A vacuolar-type H^+-ATPase that is very abundant in the ruffled membrane drives this process [Blair et al., 1989]. Acid degradation of bone creates local free calcium over 10 mM; osteoclast-bone attachment affects intracellular free-calcium activity, and extracellular free

calcium has effects on cellular activity [reviewed by Blair and Schlesinger, 1992]. As would be expected, intracellular Ca^{2+} and free calcium–regulated proteins including calmodulin are factors controlling the osteoclast's core activity, acid transport [Radding et al., 1994; Williams et al., 1996a,b).

Calmodulin is a 17 kD, 148 amino acid, calcium-binding protein, found in all eukaryotic cells; its protein sequence is completely conserved in higher vertebrates [Putkey et al., 1983]. Calmodulin binds four Ca^{2+} with bound-unbound transitions at ~20–200 nM free calcium; binding is cooperative and causes conformational changes that control binding to distinct basic-amphiphilic α-helices of target proteins. These secondary binding interactions have effective K_as in the pM-nM range and may appear or disappear with calmodulin or target molecule conformational changes [O'Neil and DeGrado, 1991]. Calmodulin binding controls many enzymes or regulatory proteins including Ca^{2+} channels, myosin, phosphodiesterases, protein kinases, and the phosphatase calcineurin [O'Neil and DeGrado, 1991]. There are other, widely diverse effects related to up- or down-regulation of many systems [Heizmann and Hunziker, 1991]. While calmodulin is present in virtually all cells, the protein is more highly expressed in some circumstances. We previously studied calmodulin in osteoclasts and determined that calmodulin concentrates at the acid-secreting membrane [Radding et al., 1994] and that protein levels are increased in osteoclasts cultured on bone [Williams et al., 1996b]. In addition, we have demonstrated that osteoclasts are highly sensitive to calmodulin antagonists, which may directly inhibit osteoclastic acid secretion [Radding et al., 1994; Williams et al., 1996a].

Tamoxifen is useful in long-term treatment for breast cancer. One of its mechanisms of action involves competitive binding with the estrogen receptor system [Coezy et al., 1982; Jordan et al., 1984]. However, tamoxifen is also an established calmodulin antagonist [Edwards et al., 1992]. We recently reported that tamoxifen inhibited osteoclastic bone resorption and H^+-ATPase activity by a mechanism independent of steroid receptors in a manner similar to other calmodulin antagonists [Williams et al., 1996a].

Here, we compare the effects of six structurally similar compounds, four benzopyrans (CDRI 85/287, cischroman, and *d*- and *l*-centchroman) and the established calmodulin antagonists tamoxifen and trifluoperazine, on osteoclastic bone degradation and two calmodulin-dependent reactions in vitro, osteoclast membrane H^+-ATPase and an unrelated reaction, calmodulin-dependent cyclic nucleotide phosphodiesterase. Direct interaction of the test compounds with calmodulin is also determined by displacement of calmodulin bound to trifluoperazine. Our results demonstrate that the six compounds all interact with calmodulin, with different effects on different calmodulin-dependent reactions, in some cases also depending on free-calcium concentration. Cellular effects have properties much less clear-cut than effects on isolated biochemical reactions, suggesting that these effects are modified in complex ways by factors such as transport across the cell membrane and differential binding to different cellular components.

METHODS

Materials

Unless specified, reagents were from Sigma (St Louis, MO). Trifluoperazine was from Calbiochem (La Jolla, CA). CDRI-85/287, centchromans, and cischroman were kindly provided by Drs. Diane Durnam and Virender Labroo (Zymogenetics, Seattle, WA).

Cell and Cell Membrane Preparations

Osteoclast-enriched cell fractions were obtained from medullary bone of laying hens, *Gallus domesticus,* using a Ca^{2+} restricted diet to increase osteoclast numbers, with cells harvested by sieving through 110 μm nylon and sedimentation through 70% serum to recover the dense osteoclasts [Blair et al., 1986]. In serum sedimented cells washed at 2 days to remove nonadherent cells, 85% of the cells were osteoclasts. Cells were incubated in Dulbecco's modified Eagle's medium [Williams et al., 1996a]. Membrane vesicles were made by nitrogen cavitation of cells followed by differential centrifugation to recover a ruffled-membrane-rich (~50%) membrane fraction [Blair et al., 1991].

Osteoclastic Bone Degradation, Protein Synthesis, and Bone Attachment

Bone resorption by avian osteoclasts was quantified using rat bone labeled in vivo with *l*-[2,3,4,5-^{3}H]-proline and milled to 20–40 μm to

provide a large surface area for cellular degradative activity [Blair et al., 1986]. Labeled bone fragments (100 µg; 25–50 cpm/µg) were added to 2–3 × 10^3 osteoclasts per 2 cm^2 tissue culture well. [^{3}H]-proline released from the labeled bone into the medium was measured relative to no-cell controls at 2–4 days, where activity is essentially linear [Blair et al., 1986] and fusion of contaminating macrophages in insignificant [Williams et al., 1996b]. Specific activity of substrate was determined by hydrolysis in 6 N HCl at 60°C for 18 h, neutralization of samples with NaOH, and liquid scintillation spectrometry.

Protein synthesis was measured by leucine incorporation in cells cultured 4 days with 25–40 µm unlabeled bone fragments in test compounds as in the bone resorption assay. Cells were incubated with 1 µCi/ml *l*-[4,5-^{3}H]-leucine (45–85 MBq/mMol) (Amersham, Arlington Heights, IL) for 6 h and washed twice with 4°C PBS. Isotope incorporated into macromolecules was precipitated 30 min at 4°C in 10% trichloroacetic acid (TCA), lipids and TCA were removed with ethanol/ether (3:1, v/v) to reduce background. Protein was solubilized by digestion in 0.1 M NaOH, and ^{3}H incorporation was measured by scintillation counting.

Bone attachment of cells was determined by adding 100 µg of 25–40 µm labeled bone to 5 × 10^3 osteoclasts at 3 days incubation, when cells are anchored to tissue culture substrate. After 4 h incubation (during which less than 2% labeled material was degraded), the wells were gently washed twice with PBS at 20°C. Retained bone was hydrolyzed in 100 µl of 6 N HCl overnight, neutralized with NaOH, and measured by scintillation counting relative to no-cell controls. Control (untreated) cells bound to and retained (anchored to the plate) ~80% of added bone fragments.

Membrane Vesicle Acid Transport

Membranes were suspended at 1.5 mg/ml protein in 120 mM KCl, 20 mM NaCl, 10 mM HEPES, pH 7.4, incubated 30 min on ice to allow vesicles to stabilize. Mg^{2+}-ATP–dependent HCl transport was determined as fluorescence quenching at 540 nm with excitation at 468 nm [Blair et al., 1991] averaged over 5 s intervals, using ~25 µg vesicle protein (10–25 µl of reconstituted vesicles) in 2.5 ml of 3.3 µM acridine orange, 1 mM ATP, 120 mM KCl, 20 mM NaCl, 10 mM HEPES, pH 7.4, at 37°C. Transport was initiated with 2 mM Mg^{2+} and measured as change after addition of a 300-fold molar excess of NH_4Cl over acridine orange, determined 15 s after NH_4Cl addition to eliminate mixing artifacts. Free calcium was set, where indicated, using 0.5 mM EGTA, pH 7.4, and the indicated free Ca^{2+} as described [Grynkiewicz et al., 1983]. EGTA greater than 0.5 mM inactivated vesicles; at 0.5 mM EGTA, control vesicles retained ~70% of no-EGTA activity after 15 min incubation. Vesicles were preincubated in assay buffer 2 min prior to initiation of the reaction with 2 mM $MgCl_2$ to control for nonspecific effects of EGTA.

Calmodulin-Dependent Cyclic Nucleotide Phosphodiesterase

Calmodulin stimulated phosphodiesterase activity was measured as the decrease in fluorescence of 2 µM 2′-(N-methyl)-anthraniloylguanosine 3′:5′-cyclic monophosphate (a fluorescent cGMP derivative) (Molecular Probes, Eugene, OR) [Johnson et al., 1987] and 8 µM unlabeled cyclic GMP during 10 min incubations at 37°C, as reported [Williams et al., 1994]. Calmodulin-independent basal activity was subtracted from all data presented. Compounds tested were preincubated 5 min with the enzyme prior to calmodulin addition. Concentration dependence of antagonists was tested at saturating calcium. Calcium dependence of calmodulin-stimulated phosphodiesterase activity was determined at the apparent half-maximal effect for that compound using calcium buffers made by varying the ratio of Ca^{2+} to EGTA, with Ca^{2+} activity measured using a selective electrode (Orion Research, Boston, MA). All compounds were diluted 1:1,000 into assay mixtures, and control comparisons used matched solvent concentrations. Background or substrate fluorescence was not measurably affected by the compounds under the conditions tested.

Trifluoperazine Sepharose and Competitive Calmodulin Binding

Trifluoperazine was coupled to CNBr activated Sepharose by the procedure reported for N-(6-aminohexyl)-1-naphthylaminesulfonamide [Endo et al., 1981]. Calmodulin binding to the resin as a function of calmodulin concentration was determined. Then, using trifluoperazine Sepharose with half-maximal bound calmodulin, displacement of bound calmodulin

by test compounds was determined as calmodulin released by increasing concentrations of test compounds, with trifluoperazine as an internal control. Trifluoperazine Sepharose (100 µl of a 1:1 slurry) was equilibrated of 25 mM Tris, pH 7.4, with 1 mM $CaCl_2$. The resin was then incubated 20 min with 1×10^6 dpm of ^{125}I-calmodulin, 1 µM unlabeled calmodulin, and the indicated concentrations of test compounds. Reactions (500 µl) were mixed 20 min at 20°C, washed four times in equilibration buffer, and displacement determined by counting ^{125}I-calmodulin remaining bound relative to controls without test compounds.

Statistics

Unless noted, means of quadruplicate determinations ± standard error are shown. Comparisons were by analysis of variance or Student's paired *t*-test as indicated, with a difference concluded if the null hypothesis is rejected at 5% confidence.

RESULTS

Radiometric assays of bone resorption by avian osteoclasts were used to compare effects of *d*- and *l*-centchroman, cischroman, CDRI 85/287, tamoxifen, and trifluoperazine (Fig. 1). Onset of effects was not immediate but developed over a few days; an example, the time course for *d*-centchroman, is shown in Figure 1A. Thus, comparisons of compounds were made using 48–72 h time points (Fig. 1B,C). Dose-dependent inhibition was obtained with *d*-centchroman, with resorption inhibited 30–40% at 1–3 µM and a half-maximal effect at ~100 nM, while *l*-centchroman inhibited only at concentrations >1 µM (Fig. 1B), a level affecting protein synthesis (Fig. 3). CDRI 85/287 and cischroman were less effective, producing 15–20% inhibition, statistically different from controls only by analysis of variance (not illustrated); inhibition by these compounds was consequently not enough to determine accurate half-maximal effects. Tamoxifen and trifluoperazine inhibited resorption 40–60% at 3–7 µM, with half-maximal effects between 1 and 3 µM (Fig. 1C). Washout experiments, measuring resorption from 3–5 days after removal of the compounds showed recovery of 90% or more of control activity when initial concentrations were less than 10 µM, indicating cell viability (not illustrated); rebound activity greater than control was not observed. Tamoxifen and trifluoperazine assays were performed using both standard and affinity purified osteoclasts as reported [Williams et al., 1996a] to assure that results are unrelated to interactions with other cells; both cell preparations gave similar results.

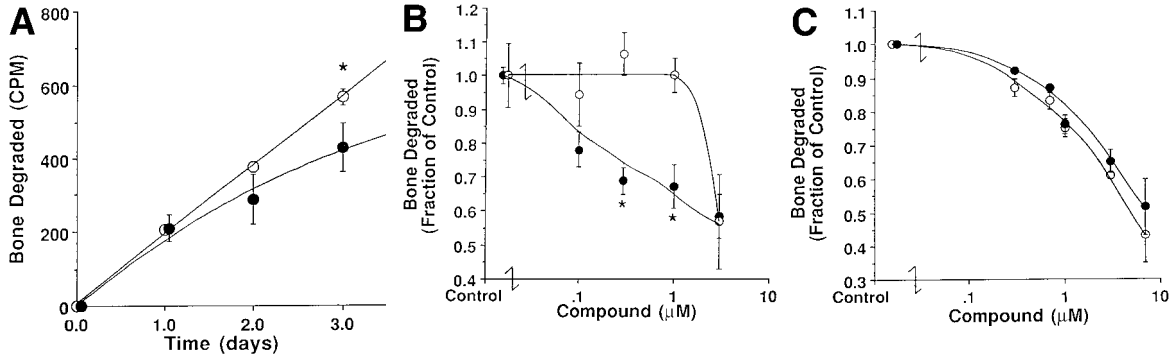

Fig. 1. Rate of bone resorption by avian osteoclasts as functions of inhibitor time and concentration. Bone resorption was determined as label released into supernatants by 2×10^3 osteoclasts from 100 µg [^{3}H]-proline bone fragments, with no-cell controls subtracted. Data are quadruplicate determinations ± SEM and are representative of three experiments. Concentration effects (B,C) are expressed as percent of control degradation over 2 days after a 1 day preincubation; control degradation was ~15% of total substrate during this period. Asterisk indicates $P < 0.05$ relative to control by the Student's *t*-test. Note that logarithmic concentration scales are used, here and in following figures, causing variance in curve profiles relative to earlier work referenced [Williams et al., 1996a]. Cischroman and CDRI 85/287 effects were too small for reliable determinations (10–15% at 3 µM) and are not illustrated. **A:** Time course comparing bone resorption in 1 µM *d*-centchroman (closed circles) to control medium (open circles), showing the delayed onset of inhibition. **B:** Bone resorption relative to *d*- or *l*-centchroman concentration (closed and open circles, respectively). **C:** Bone resorption relative to tamoxifen (open circles) or trifluoperazine (closed circles) concentration. With these compounds, all concentrations shown gave results different from controls by the *t*-test.

However, since washout results might reflect reversible toxic cellular effects rather than nonspecific effects, assays for bone attachment and protein synthesis were also performed. To determine whether inhibition of resorption reflected differences in cell attachment to bone, freshly harvested cells were cultured 4 h with labeled bone (Fig. 2). Under these conditions, control cells bind ~80% of the cpm in 100 µg of added bone (~4,000 of 5,000 cpm/well). No statistically meaningful differences in cell attachment were noted with the benzopyran compounds at concentrations below 10 µM. Bone binding by osteoclasts in 10 µM *d*-centchroman was inhibited ~50% (Fig. 2). We measured 6 h ^{3}H leucine incorporation in cells incubated 4 days in the presence of *d*- and *l*-centchroman, cischroman, and CDRI 85/287 to determine whether the compounds affected protein synthesis (Fig. 3). None of the compounds inhibited ^{3}H leucine incorporation into trichloroacetic acid–insoluble material at concentrations below 10 µM. However, significant inhibition was seen with *d*-centchroman, CDRI 85/287, and cischroman at 10 µM. *l*-centchroman had no adverse effect on protein synthesis; on the contrary a modest increase in ^{3}H leucine incorporation was seen at ≥1 µM, the meaning of which is unclear.

The central metabolic activity of the osteoclast is secretion of HCl into the extracellular space at the matrix-degradation site. This calmodulin-sensitive acid secretion process is retained in isolated cell membranes. We determined the effect and concentration dependence of each compound on HCl transport in vesicles by measuring acridine orange uptake as ATP-dependent fluorescence quenching (Fig. 4A). Surprisingly, all of the compounds inhibited vesicle acidification completely, with *l*-centchroman producing essentially the same result as *d*-centchroman. The IC_{50} of tamoxifen has been reported as ~250 nM, while trifluoperazine is between 1 and 2 µM [Williams et al., 1996a]. The other dose dependencies were generally close to that of tamoxifen, with half-maximal effects at ~300 nM. The membrane assay thus shows a similar dose-response range to the whole cell assay, but there is an obvious difference in degree of effect. At nontoxic levels, maximal inhibition of osteoclastic bone resorption was ~50%. The cell membrane ATP-dependent acid transport assay bypasses cellular permeability and most cellular feedback mechanisms;

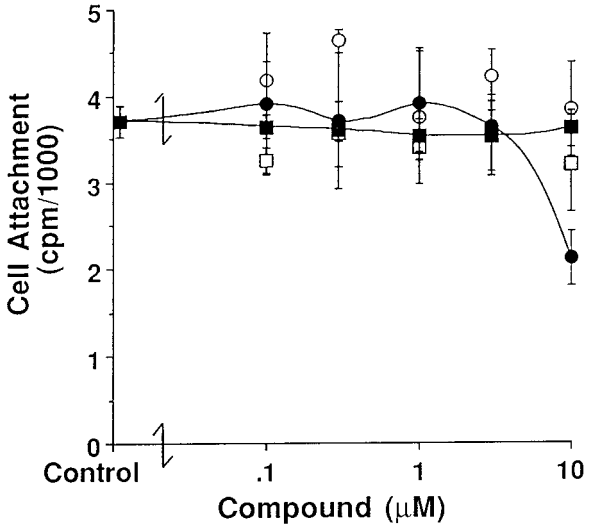

Fig. 2. Cell attachment to bone as a function of inhibitor concentration. Four hour binding of 100 µg of labeled bone (~5,000 cpm) was determined at indicated concentrations of *l*-centchroman (open circles), *d*-centchroman (closed circles), cischroman (open squares), and CDRI 85/287 (closed squares). Statistically significant effects at concentrations below 10 µM were not seen (note inhibition in *d*-centchroman). Results for tamoxifen and trifluoperazine were similar, with no inhibition below 10 µM [Williams et al., 1996a]. Data represent one quadruplicate experiment, mean ± SEM.

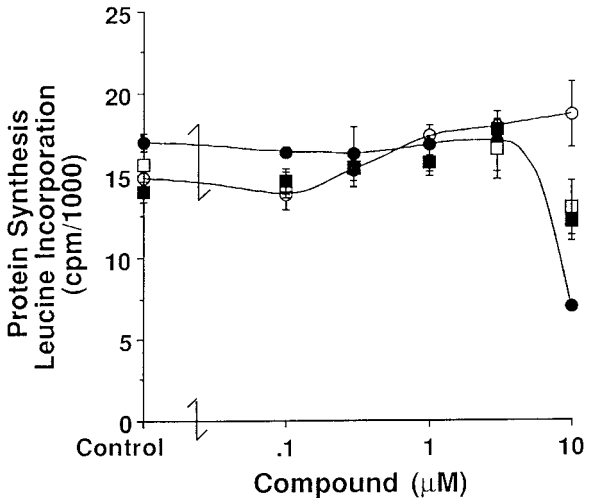

Fig. 3. Effects of test compounds on protein synthesis. Six hour incorporation of *l*-[4,5-^{3}H]-leucine into TCA-precipitable material was measured in cells incubated in indicated concentrations of *l*-centchroman (open circles), *d*-centchroman (closed circles), cischroman (open squares), and CDRI 85/287 (closed squares) for 4 days. Data are expressed as percent of controls less no-cell blanks to facilitate comparisons. Label incorporation was reduced at 10 µM for all compounds but *l*-centchroman, which showed a modest increase in precipitable label. Results for tamoxifen and trifluoperazine were variably inhibited at 10 µM [Williams et al., 1996a]. Data represent one quadruplicate determination ± SEM.

these factors may explain the complete inhibition (see Discussion).

The results to this point were consistent with a mechanism involving calmodulin-dependent inhibition of osteoclastic activity by the analogs tested. To confirm this in a system with fewer confounding variables, we used calmodulin-dependent cyclic nucleotide phosphodiesterase. Hydrolysis of a fluorescent cGMP derivative by 4 nM phosphodiesterase in 100 nM calmodulin was measured as a function of concentration for each substance (Fig. 5A). This demonstrated nearly complete inhibition by all compounds, as in the vesicle acidification assay, but effective concentrations and relative potencies diverged widely from those observed in bone resorption or vesicle acidification assays, with half-maximal effects of ~5 μM for *d*- and *l*-centchroman, ~2 μM for CDRI 85/287, ~7 μM for cischroman, and >7 μM for trifluoperazine (Fig. 5A) and ~2 μM for tamoxifen [Williams et al., 1996a]. These values average tenfold greater than inhibitory levels in bone resorption or vesicle acidification assays (Fig. 4A). However, *d*- and *l*-centchroman inhibited the phosphodiesterase assay at similar concentrations, as had been noted in the vesicle acidification assay, a surprising result in light of their divergent effects on bone resorption (Fig. 1B).

An important parameter that may cause differential sensitivity in vesicle acidification or phosphodiesterase assays relative to intact cell assays is the concentration of free calcium. In cells, average calcium is on the order of 100–500 nM, while in experiments to this point free calcium was not specifically controlled and was estimated to be 10–20 μM, a level precluding any additional calcium-dependent variation in calmodulin conformation or free calcium–calmodulin binding. To test whether the effects of the tamoxifen-like compounds were calcium-dependent, we compared the antagonistic effects of *d*- and *l*-centchroman as a function of free calcium on vesicle acidification (Fig. 4B,C) using Ca^{2+}-EGTA buffers (see Methods). Vesicle acidification assays were conducted in the presence of 300 nM *d*- or *l*-centchroman at a free Ca^{2+} of 330 nM (Fig. 4B) or 17 μM (Fig. 4C). The effect of *d*-centchroman varied with free calcium, while *l*-centchroman had similar effects at both nanomolar and micromolar free calcium. Unfortunately, vesicle activity was not retained at still lower free calcium using this buffer system, precluding measurement of the

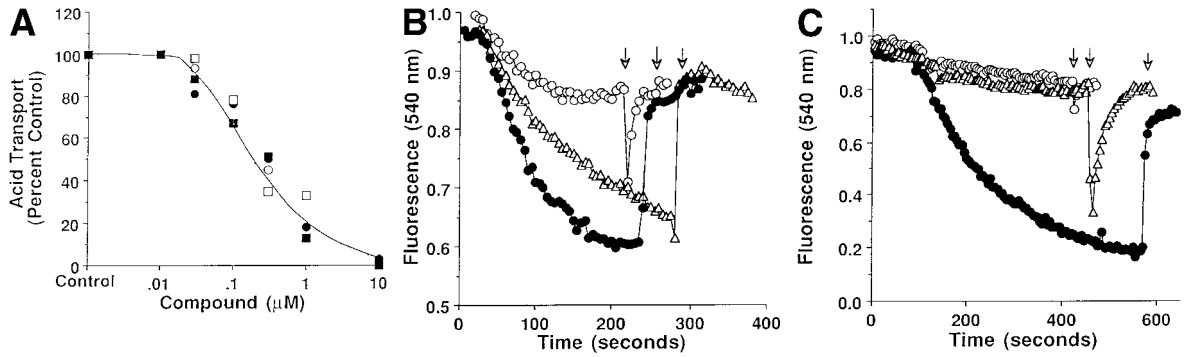

Fig. 4. Effects of test compounds on ATP-dependent acid transport in osteoclast membrane vesicles. Acid transport was measured, with acridine orange as an indicator, as fluorescence quenching at 540 nm with excitation at 468 nm, using 25 μg vesicle protein in 2.5 ml assay buffer with the indicated concentrations of test compounds. Transport was initiated with 2 mM Mg^{2+} and quantified by fluorescence difference on acridine orange washout with excess NH_4Cl, relative to control. Transient changes on addition of NH_4Cl in B and C are mixing artifacts. Note that the experiments shown in B and C are entirely different, including the controls, and these are graphed on different scales to illustrate clearly the differences. **A:** Concentration dependence of *l*-centchroman (open circles), *d*-centchroman (closed circles), cischroman (open squares), and CDRI 85/287 (closed squares) effects. Data are means of duplicate assays. **B:** Effect of *d*- and *l*-centchroman at low levels of free calcium. Transport was measured in buffers containing 500 μM EGTA at 330 nM free Ca^{2+}, calculated as reported [Grynkiewicz et al., 1983] (control; closed circles) and with addition of 300 nM *d*- or *l*-centchroman (open triangles and circles, respectively). Arrows indicate addition of NH_4Cl. Representative results of duplicate experiments are shown. **C:** Effect of *d*- and *l*-centchroman on membrane vesicle acid uptake at saturating free-calcium activity. Transport was measured in buffer containing 0.5 mM EGTA with 17 μM free Ca^{2+} alone (control; closed circles) or in assays containing 300 nM *d*- or *l*-centchroman (open triangles and circles, respectively). Arrows indicate addition of NH_4Cl. Data are representative of results from duplicate experiments; *d*- and *l*-centchroman were also similarly effective as inhibitors at saturating free calcium in buffers that did not contain EGTA (A).

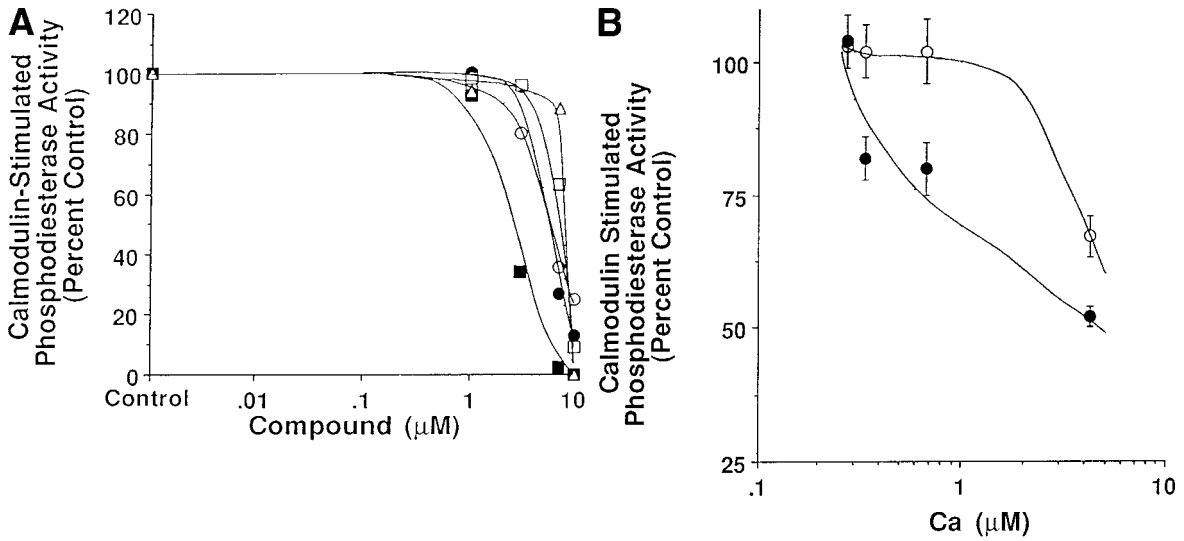

Fig. 5. Effects of test compounds on phosphodiesterase activity. Hydrolysis of 2′-(N-methyl)-anthraniloylguanosine 3′:5′-cyclic monophosphate in 10 min assays at 37°C was measured as fluorescence decrease at 450 nm with excitation at 280 nm. **A:** Concentration dependence of test compounds on phosphodiesterase activity. The effect of the indicated concentrations of *l*-centchroman (open circles), *d*-centchroman (closed circles), cischroman (open squares), CDRI 85/287 (closed squares), and trifluoperazine (triangles) on cyclic nucleotide-dependent phosphodiesterase activity was determined. **B:** Free-calcium dependence of phosphodiesterase inhibition by *l*-centchroman (5 μM, open circles) and *d*-centchroman (5 μM, closed circles). Phosphodiesterase activity is shown as a fraction of control in the same Ca^{2+} buffer. Free calcium was controlled by Ca^{2+}-EGTA buffers (see Methods). Other test compounds did not differentially affect phosphodiesterase activity and are not illustrated.

effect at 0–300 nM calcium. Similarly, calmodulin-stimulated phosphodiesterase activity was inhibited by 5 μM *d*-centchroman at 250 and 400 nM free calcium, while in similar experiments with 5 μM *l*-centchroman inhibition was seen only at micromolar free calcium (Fig. 5B). This indicates that some disparities in calmodulin dependence of in vitro and in vivo reactions may be attributable to differences in the concentration of free Ca^{2+}. However, free calcium–dependent effects were not noted with cischroman and CDRI 85/287.

The calmodulin-dependent phosphodiesterase assay strongly suggested that these compounds act as calmodulin antagonists but did not rule out indirect effects of the compounds, such as action on the phosphodiesterase itself. Therefore, we assessed the ability of each compound to displace ^{125}I-calmodulin from immobilized trifluoperazine. Trifluoperazine Sepharose was synthesized (see Methods) and [^{125}I]-calmodulin binding determined (Fig. 6A). Subsequently, displacement of [^{125}I]-calmodulin by each compound was determined as a function of concentration. As hypothesized, all of the compounds displaced calmodulin (Fig. 6B); all of the benzopyrans had effects at concentrations below 1 μM (column 4, Table I). Deviations from sigmoidal traces on these semilogarithmic plots may in part represent experimental error and in part nonideality of interactions.

DISCUSSION

Calmodulin-ligand interactions are important to the control of a wide variety of cellular mechanisms [Putkey et al., 1983; O'Neil and DeGrado, 1991; Heizmann and Hunziker, 1991]. A direct consequence of this is that specific inhibition of a single cellular function or of a limited range of cellular activities by a calmodulin antagonist is difficult to obtain. However, the wide utility of compounds such as phenothiazines [Motohashi, 1991], which are well-characterized calmodulin antagonists used in neuropsychiatric disorders, argues that there must be some selectivity of calmodulin antagonists for specific calmodulin-dependent interactions. We compared the effects of trifluoperazine, tamoxifen, and several structurally similar benzopyrans on avian osteoclastic bone resorption and its key calmodulin-dependent transport process, ruffled membrane acid transport, together with assays of calmodulin-dependent cyclic nucleotide phosphodiesterase and direct calmodulin displacement from immobilized trifluoperazine. The results of these assays are summarized in Table I.

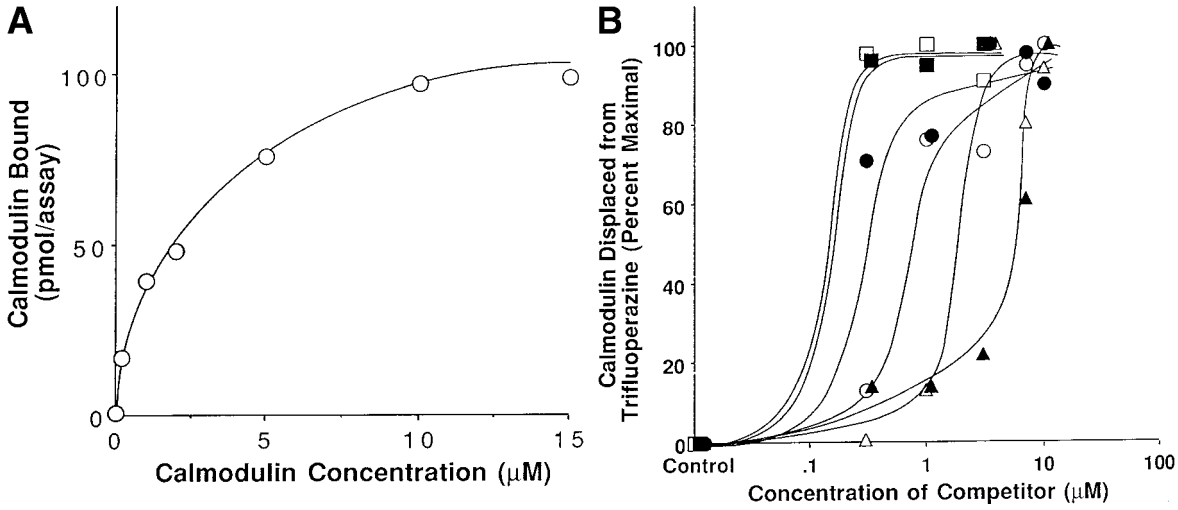

Fig. 6. Tamoxifen-related compounds compete for binding with calmodulin. Trifluoperazine Sepharose (100 µl of a 1:1 w:v aqueous slurry) was equilibrated in 25 mM Tris, pH 7.4, with 1 mM $CaCl_2$, as described in Methods. Resin was incubated with 1×10^6 dpm labeled calmodulin, 1 µM unlabeled calmodulin, and the indicated concentrations of test compounds. The reactions (500 µl) were rotated 20 min at room temperature and washed four times in equilibration buffer. Bound [^{125}I]-calmodulin was determined by centrifuging the resin, aspirating the wash buffer, and counting the pellet. Data are means of duplicate determinations, with ranges ~10% of reported values. These are not illustrated to maintain clarity with several data sets displayed together. **A:** Binding of calmodulin to trifluoperazine Sepharose as a function of calmodulin concentration. Unlabeled calmodulin at indicated concentration and labeled calmodulin (2 pM) were present during equilibration. Half-maximal binding is at 1 µM, which was used in subsequent assays. **B:** Competition of *l*-centchroman, *d*-centchroman, tamoxifen, cischroman, CDRI 85/287, and trifluoperazine (internal control) for binding with trifluoperazine-bound calmodulin. Bound label without addition is defined as zero (control). Maximum displacement was ~50% of total pellet counts. *l*-centchroman (open circles) displaces trifluoperazine at 0.7 µM; *d*-centchroman (closed circles) displaces trifluoperazine at 0.3 µM and tamoxifen (open triangles) at 2 µM. Cischroman (open squares) and CDRI 85/287 (closed squares) both displace trifluoperazine at <0.3 µM under these conditions. Trifluoperazine displaced itself only at relatively high concentrations, 7 µM (closed triangles).

TABLE I. Properties of Tested Compounds in Bone Resorption, Acid Secretion, Calmodulin-Sensitive Phosphodiesterase Activity, and Displacement of Calmodulin-Trifluoperazine Binding Assays*

Compound	IC_{50} (µM)			Calmodulin displacement (µm)
	Bone resorption	H^+-ATPase	Phosphodiesterase	
d-centchroman	0.1	0.3	~5	0.7
l-centchroman	>1.0	0.3	~5	0.3
Cischroman	ND	0.3	~7	<0.3
CDRI 85/287	ND	0.3	2	<0.3
Trifluoperazine	1–3	1–2	>7	2
Tamoxifen	1–3	~0.3	2	7

*Effects are expressed as concentration giving 50% inhibition (H^+-ATPase or phosphodiesterase) or half-maximal effect for the cellular assay, maximal being the effect at the highest concentration at which cell recovery occurred after washout. ND, effect not large enough for a reliable determination (see Fig. 1).

All of the compounds tested inhibited osteoclasts at least slightly, but effects varied widely (Fig. 1B,C). Tamoxifen, *d*-centchroman, and trifluoperazine were reasonably effective inhibitors, with maximum cellular inhibitory effects on the order of 50% at concentrations not significantly affecting cellular protein synthesis or cell attachment (Figs. 2, 3). As expected, *l*-centchroman had effects different from *d*-centchroman. No convincing cellular inhibition was noted in *l*-centchroman below 3 µM (Fig. 1B). The differential effects of the isomers of centchroman on osteoclasts are the opposite of those observed on the estrogen receptor [Edwards et al., 1992; Ray et al., 1994]. These differences are discussed in more detail below, but it should be

noted that the preparations of mature osteoclasts used are not estrogen-responsive [Williams et al., 1996a]. Bone resorption activity differentiated the stereoisomers of this compound, but the phosphodiesterase or H^+-ATPase assays did not (Figs. 4, 5), suggesting that the cellular assay is more sensitive to conformational differences of test compounds or that the processes affected are complex and regulatory mechanisms are intact in the whole cell assay. Cischroman and CDRI 85/287 were poor inhibitors in the cellular assay but excellent inhibitors in the subcellular ATP-dependent acid transport assay (Fig. 4A) and phosphodiesterase assays (Fig. 5A). These assays also showed relatively sharp concentration dependences, suggesting cooperative interactions, which are entirely consistent with previous analysis of calmodulin binding by low molecular weight compounds. Differences in extracellular and intracellular concentrations, competition by other intracellular reactions, and cellular feedback may contribute to these variations, although specific mechanisms underlying differences in sensitivity of cellular and noncellular assays cannot be established. Under some circumstances, pit resorption assays might differentiate effects difficult to resolve using radiolabeled substrate, such as cischroman and CDRI 85/287. This was tested, but assay variability was higher in pit resorption assays [Williams, McDonald, and Blair, unpublished observations], and the data are not reported in this study.

While the H^+-ATPase assay is complex, and inhibition by micromolar levels of the benzopyrans including *d*- and *l*-centchroman might be attributed to nonspecific reactions unrelated to calmodulin, it was surprising that *d*- and *l*-centchroman were also both antagonists in the well-characterized calmodulin-dependent cyclic nucleotide phosphodiesterase assay (Fig. 5A) where only one protein in addition to calmodulin is present. The centchroman stereoisomer pair was included in the study specifically as a control; it was expected that one would be inactive. All of the test compounds inhibited phosphodiesterase activity with 50% inhibition at 1–10 μM; 50% inhibition by *d*- or *l*-centchroman was at similar concentrations. While unexpected, these results are in keeping with reported strong interactions of calmodulin with *d* and *l* peptides [Fisher et al., 1994]. However, neither the phosphodiesterase nor the H^+-ATPase assays directly establish that the test compounds interact with calmodulin. However, competition with trifluoperazine-calmodulin binding demonstrated that all of the compounds displace calmodulin at micromolar levels (Fig. 6B). These data indicate that direct calmodulin binding plays a role in the observed effects, while other mechanisms may also be involved. Nonphysiologic conditions may contribute to the observed results. The phosphodiesterase assay used 100 nM calmodulin, 1/10 to 1/100 of the test compound levels, and only one calmodulin-binding protein, the phosphodiesterase, was present. The concentration of the phosphodiesterase, 4 nM, was 1/250 to 1/2,500 the apparent half-maximal effects of the test compounds. These conditions all favor the binding of calmodulin to the test compounds.

Further, both the H^+-ATPase and phosphodiesterase assays were run at saturating free calcium, which would strongly favor binding. High free-calcium concentrations maximize the proportion of calmodulin in its calcium-saturated form, and calcium-bound calmodulin is known to assume binding conformations encompassing significant variation in its hydrophobic pocket, as indicated by differences reported in separations of amino acid residues in calmodulin-trifluoperazine complexes [Cook et al., 1994] compared to the same measurements in calmodulin complexed with the calmodulin-binding subunit of calmodulin kinase [Meador et al., 1993] or the calmodulin-binding domain of rabbit skeletal myosin light chain kinase in solution [Ikura et al., 1992]. Additionally, concentrations of test compounds were equimolar to 100-fold in excess of the calmodulin concentration and thus strongly favored competitive binding. In vivo, the added constraints of free calcium binding equilibrium and competition by many natural substrates would be expected to increase the selectivity of calmodulin binding relative to artificial inhibitors with marginal conformational similarity, and large molar excesses of competing artificial ligands relative to natural ligands would be unlikely in living cells.

It was hypothesized that different responses to *d*- and *l*-centchroman in the cellular, membrane transport, and phosphodiesterase assays (Table I) might reflect, in part, differences in free calcium. Assays were performed using Ca^{2+}-EGTA buffers to model intracellular free calcium. Under these conditions, the assays discriminated *d*- and *l*-centchroman effects (compare Figs. 4B and 5B). This suggests that

inhibition of calmodulin-dependent processes is more selective at intracellular calcium levels, although other variables are probably involved. *l*-centchroman inhibited both acid transport and phosphodiesterase activity at low or high calcium, in contrast to observations in the cellular assay. *d*-centchroman antagonist activity varied with free calcium at physiologically relevant levels. The full meaning of these observations will require structural analysis beyond the scope of the present work. Possibilities include that alternative binding sites on calmodulin may be involved, depending on the competing ligands and concentration of free Ca^{2+}, as suggested by conflicting reports of calmodulin antagonist binding stoichiometry [Cook et al., 1994; Vandonselaar et al., 1994]. Variable calmodulin antagonist effects in different assays is not surprising. Calmodulin interactions are heterogeneous, and when calmodulin binds amphiphilic peptides its structure is altered. The deformation of calmodulin may cause a spectrum of changes in reactivity [James et al., 1995]. Our results suggest that it is important to test calmodulin reactions at physiological calcium when such interactions are studied with the object of modeling physiological interactions, because some interactions, such as those of *d*- and *l*-centchroman, differ dramatically depending on calcium concentrations.

From the structural similarities of the tested compounds and the known effects of tamoxifen and trifluoperazine on calmodulin, it was expected that the tested compounds would affect calmodulin-dependent processes. All of the compounds tested contain ring structures and polar side chains, although the dimensions differ and properties such as conformation vary in ways that are difficult to predict, as seen by comparison of calmodulin-antagonist modeling and direct structural determinations [Salman et al., 1986]. All of the compounds have flexible polar side chains, contributing to this uncertainty. From this perspective, the differences in antagonist properties between antiestrogen effects and calmodulin-binding effects are also unsurprising. Structural modeling of estrogen and *l*-centchroman suggested that *l*-centchroman would be the biologically active enantiomer for the estrogen receptor [Ray et al., 1994], and in fact *l*-centchroman has a sevenfold greater estrogen receptor binding affinity [Salman et al., 1986], although both enantiomers have complex estrogen agonistic and antagonistic activity. Our findings suggest that in osteoclasts the *d* enantiomer is more reactive. Here, interactions with estrogen receptors are probably unimportant; mature osteoclasts do not respond directly to estrogen, and, in keeping, tamoxifen affects osteoclasts by disrupting calmodulin-dependent processes similarly to other calmodulin antagonists [Williams et al., 1996a]. Comparison of the calmodulin antagonistic properties of the *d* and *l* enantiomers of centchroman is unique to this work. Confirmatory displacement studies with these compounds support the hypothesis that these compounds directly interact with calmodulin, under these in vitro conditions, with half-maximal displacement of ^{125}I-calmodulin from trifluoperazine Sepharose between 1 and 10 μM (Fig. 6B).

The cellular assay results and concentration dependencies are consistent with other results showing osteoclast inhibition by (*d*, *l*)-centchroman [Hall et al., 1995], tamoxifen [Turner et al., 1988; Williams et al., 1996a], and trifluoperazine [Radding et al., 1994]. The report of 70% inhibition of rat osteoclast pit formation by 1 μM (*d*, *l*)-centchroman [Hall et al., 1995] resembles our radiochemical resorption result for the *d*-isomer in avian cells (half-maximal effect ~0.1 μM, 40% inhibition at 1 μM), showing that these two models respond similarly to this compound. This would be the expected for calmodulin-controlled processes, which are conserved. Large numbers of purified cells allowed us to study bone attachment and cellular protein synthesis, which showed no inhibition at $<$10 μM (Figs. 2, 3), and recovery of cellular activity on washout of test compounds. These results, and the inhibition of acid transport at doses similar to those affecting cellular activity (Fig. 4A), suggest that inhibition of cellular acid secretion may be a major target of activity of this class of compounds and conversely indicate that the effects observed are not related to general cellular toxicity. Osteoclastic function depends centrally on massive expression of a vacuolar-class H^+-ATPase at the cell's bone attached surface, and acid secretion is maintained by calmodulin [Radding et al., 1994; Williams et al., 1996a], in keeping with a calmodulin-dependent mechanism for the effects observed here. Our findings are also consistent with our work indicating that interaction of tamoxifen with osteoclasts depends on membrane interactions [Williams et al., 1996a]. On the other hand, other cell types may respond by

alternative mechanisms, and unrelated effects on other cellular processes in the osteoclast probably also exist.

None of the compounds tested abolished osteoclastic bone resorption at doses maintaining cellular viability, and whether they will be useful in vivo is not clear. However, bone formation and degradation are rarely unbalanced by more than 5–10% even when rapid bone loss occurs, so that partial inhibitors such as bisphosphonates are clinically useful. Direct measurements in animals and humans have shown that tamoxifen reduces bone turnover, improving mineral balance without severe effects, such as hypocalcemia, which would result from profound inhibition of osteoclasts [Turner et al., 1988; Wright et al., 1993]. These observations are consistent with the effects of tamoxifen that we observed in vitro. If effects of the additional compounds tested also occur in vivo to the degree observed in vitro, they may provide similarly useful therapeutic effects.

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