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Novel Ipriflavone Receptors Coupled to Calcium Influx Regulate Osteoclast Differentiation and Function

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ABSTRACT

Ipriflavone (7-isopropoxyisoflavone) is an effective antiresorptive agent used to treat osteoporosis. However, the mechanism of its action on osteoclasts and their precursor cells is not well understood. To determine whether the mechanism involves direct effects on osteoclasts or their precursors, we examined the effects of ipriflavone on cytosolic free calcium ($[Ca^{2+}]_i$) in osteoclasts and their precursors and measured specific binding of ^{3}H-labeled ipriflavone. Highly purified chicken osteoclast precursors, which spontaneously differentiate into multinucleated osteoclasts in 3–6 days, were loaded with fura-2, and the subcellular $[Ca^{2+}]_i$ distribution was monitored by videoimaging. Ipriflavone induced a rapid increase in $[Ca^{2+}]_i$ followed by a sustained elevation [$EC_{50} = 5 \times 10^{-7}$ M, 263 ± 74 nM (SE) (n = 8) above basal levels, by 10^{-6} M ipriflavone, sustained phase]. The responses were the same in differentiated chicken osteoclasts and isolated rabbit osteoclasts. An influx of extracellular Ca^{2+} is likely to be responsible for the ipriflavone-induced change in $[Ca^{2+}]_i$ because the response was abolished by 0.5 mM $LaCl_3$, or by Ca-free medium containing EGTA. Moreover, high $[Ca^{2+}]_i$ levels were detected adjacent to the cell membrane after ipriflavone addition. Ipriflavone induced Ca influx mainly through dihydropyridine-insensitive Ca^{2+} channels, because nicardipine (10^{-7} M) and verapamil (10^{-7} M) had no effects on ipriflavone-induced $[Ca^{2+}]_i$ responses. [^{3}H]Ipriflavone binding studies indicated the presence of specific ipriflavone binding sites (two classes), both in precursor cells [dissociation constant (K_d), 7.60×10^{-8} M, 2.67×10^{-6} M] and in mature osteoclasts (K_d, 4.98×10^{-8} M, 3.70×10^{-6} M). Specific ipriflavone binding was not displaced by various modulators of avian osteoclast function, such as estradiol (10^{-6} M) or retinoic acid (10^{-6} M), indicating that ipriflavone receptors differ from the receptors for these Ca-regulating hormones. The fusion of osteoclast precursor cells was significantly inhibited by ipriflavone, which led to dose-dependent inhibition of bone resorption and tartrate-resistant acid phosphatase activity. Novel specific ipriflavone receptors that are coupled to Ca^{2+} influx were demonstrated in osteoclasts and their precursor cells. These ipriflavone receptors may provide a mechanism to regulate osteoclast differentiation and function. (*Endocrinology* **137:** 3544–3550, 1996)

IPRIFLAVONE (7-isopropoxyisoflavone) is an effective antiresorptive agent used to treat osteoporosis (1, 2). The detailed mechanisms by which ipriflavone inhibits osteoclastic activity remain unclear. We suggested that ipriflavone inhibits pit formation by mature osteoclasts in mixed cell cultures of mouse bone (3). Ipriflavone also inhibits osteoclast formation in murine stromal cells cocultured with spleen cells (4). One explanation for this is indirect action through other cell types, because ipriflavone acts on cells of osteoblast lineage (5, 6). We reported ipriflavone stimulation of osteoblast differentiation and enhancement of calcification in rat stromal cell culture (5). Similar processes have been found by Cheng *et al.* (6), in human osteoblast culture. These data suggest that ipriflavone directly affects osteoblasts and enhances their ability to coordinate bone remodeling. Also, the inhibition of prostaglandin E_2 (PGE_2) production in stromal cells by ipriflavone may explain why ipriflavone decreased osteoclast formation (4).

On the other hand, histochemical and ultrastructural studies (7) have shown that rats treated with ipriflavone developed abnormalities of mature osteoclasts immediately, suggesting a direct action of ipriflavone on osteoclast lineage cells.

The aim of this study was to clarify whether ipriflavone has direct actions on mature osteoclasts as well as on their precursor cells. Because increases in cytosolic calcium, $[Ca^{2+}]_i$, inhibit osteoclast adherence and activity (8–10), we first examined the effects of ipriflavone on $[Ca^{2+}]_i$ of osteoclasts and their precursor cells by single-cell Ca imaging. We used the chicken osteoclast differentiation model (11–14), in which mononuclear monocyte-macrophage lineage cells spontaneously fuse and differentiate into multinucleated osteoclast-like cells that produce pits and have many characteristics of osteoclasts, such as tartrate-resistant acid phosphatase (TRAP) and vitronectin receptors (11). These multinucleated osteoclast-like cells in culture cannot be distinguished from native osteoclasts. This system has an advantage over other differentiation models such as coculture of stromal cells and spleen cells (4). Pure populations of precursor cells can be generated with which to study osteoclast differentiation biochemically and on a molecular basis. Furthermore, the ability to prepare large number of pure cells in this chicken osteoclast maturation model enabled the binding of ^{3}H-labeled ipriflavone to be studied. This study showed that ipriflavone binding coupled with the Ca influx

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pathway is closely correlated with the inhibition of TRAP and the bone resorptive activity of differentiating osteoclasts.

Materials and Methods

Materials

Ipriflavone was synthesized and supplied by Takeda Chemical Industries (Osaka, Japan). 7-[1′,2′-[^{3}H]ipriflavone was obtained from Nemoto Co. Ltd. (Tokyo Japan). The acetoxymethyl ester of fura-2 was purchased from Dojin (Kumamoto, Japan). Bay K8644 was from Calbiochem (La Jolla, CA). All other reagents were of analytical grade and were from Sigma Chemical Co. (St. Louis, MO).

Preparations of chicken osteoclast precursors

Osteoclast precursors were prepared as reported (11–14), whereby virtually pure preparations of multinucleated putative osteoclast-like cells were generated in culture from mononuclear cell precursors. Briefly, medullary bone from femurs and tibias of calcium-deficient laying hens were removed, rinsed, and filtered through a 100-μm nylon sieve. The filtrate was centrifuged for 5 min at 300 × g. The cell suspension was centrifuged at 380 × g for 30 min on Ficoll-Hypaque in PBS (density 1.077). The cells from the interface of the Ficoll-Hypaque gradient centrifugation were cultured in α-MEM containing antibiotics and 5% each FCS (GIBCO, Grand Island, NY) and chicken sera (GIBCO) in 150-mm plastic dishes (Falcon 3025; Becton Dickinson, Lincoln Park, NJ) at 2 × 10^8 cells/plate at 5% CO_2 and 39 C. After 18–24 h, nonadherent cells were separated and resuspended in the culture media used above with 5 μg/ml cytosine-D-arabinofuranoside and replated at 5 × 10^6 cells/ml. The multinucleated cells formed by this technique formed resorption pits in bone slices and resorbed bone particles as described (11, 14).

Rabbit osteoclast culture

Cells isolated from rabbit long bones were plated onto glass coverslips at a density of 1 × 10^6 cells/ml (15). After culture for 3 h, the medium was exchanged for fresh medium. On the following day, cells were purified by pronase digestion as described (15), and $[Ca^{2+}]_i$ was studied (10).

Measurement of $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ imaging

$[Ca^{2+}]_i$ was measured in single cells using the fluorescent calcium indicator, fura-2. Cells were washed with PBS and loaded with 10 μm acetoxymethyl ester of fura-2 for 1 h at 25 C in Krebs-Ringer HEPES (KRH) buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 25 mM HEPES, 6 mM glucose). After two washes with KRH buffer, the measuring chamber was mounted to a microfluorometric system and images (F_{340},F_{360}) were collected at wavelengths of 340 and 360 nm. The ratio images of F_{340}/F_{360} constructed after background image subtraction disclosed subcellular $[Ca^{2+}]_i$ localization, and average cell $[Ca^{2+}]_i$ values were calculated from calibration curve as described (16, 17).

Measurement of cyclic AMP (cAMP) production

Nonadherent chicken osteoclast precursors were seeded in 24-well plates (Falcon) at a density of 4 × 10^6 cells/well. On day 6, multinucleated osteoclast-like cells were washed twice with PBS, then incubated with 1 mM isobutyl methylxantine containing serum-free α-MEM for 1 h. The medium was replaced with serum-free α-MEM containing either ipriflavone or PGE_2 or control vehicle (ethanol, final concentration 0.01%). After 10 min, the medium was aspirated and 0.5 ml of 6% trichloracetic acid was added. Cells were sonicated and trichloracetic acid was removed with ether. Cellular cAMP was measured by RIA as described (18).

[^{3}H]Ipriflavone binding assay

The [^{3}H]Ipriflavone binding was assayed as described by Masuyama *et al.* (19) with some modification. Osteoclast precursors or mature osteoclasts (1 × 10^4 cells/well) were incubated at 37 C for 3 h with 1 × 10^{-8} M (about 1.5 × 10^5 dpm) [^{3}H]ipriflavone and various unlabeled agents in 1 ml of culture medium consisting of α-MEM containing 5% FCS. The [^{3}H]ipriflavone binding was at apparent equilibrium under these conditions. Binding was terminated by aspirating the medium and washing the cells with ice-cold PBS four times. One milliliter of ethanol was added to the cells; after 2 h at room temperature, the radioactivity levels in the ethanol were measured. The specific [^{3}H]ipriflavone binding was calculated by subtracting the nonspecific binding in the presence of excess unlabeled ipriflavone. Scatchard analysis of binding data was performed using the computer program described by Ikeda *et al.* (20).

Assay of cellular TRAP activity

The cellular TRAP activity was assayed as described by Sinha *et al.* (21). The cell layers were washed twice with PBS, scraped into 1 ml 0.2% Nonidet P-40, and sonicated in an ice bath for 30 sec using an ultrasonic disruptor (Tomy Seiko Co. Tokyo, Japan). The cell lysates were centrifuged at 3000 rpm for 10 min, and the supernatant was assayed. The reaction mixture (1 ml) consisted of 0.1 M sodium acetate (pH 5.0), 1 ml $MgCl_2$, 50 mM sodium tartrate and 10 mm p-nitrophenylphosphate as the substrate and an appropriate amount of the supernatant (10–50 μl). The reaction mixture was incubated at 37 C for 30 min during which the reaction proceeded in a linear fashion. The reaction was stopped by adding 1 ml of 0.2 N NaOH, and the amount of p-nitrophenol was measured in terms of the absorbance at 405 nm. Protein was determined by the method of Bradford (22), and enzyme activities were expressed as nanomoles p-nitrophenol per minute per milligram protein.

Measurement of bone resorption

Bone resorption was assessed by measuring the amount of radioactivity released into the medium after adding ^{3}H-labeled bone particles to the cultured cells (11, 14). Osteoclast precursor cells were plated at a density of 2 × 10^6 cells/ml into 48-wells plate (Costar, Cambridge, MA). Bone particles (25–63 μm diameter) were added at day 2 of culture, and after 24 h, aliquots of supernatant (0.8 ml) were removed and the ^{3}H content was measured.

Statistical analysis

Statistical comparisons were made by one-way analysis of variance (ANOVA). When significant effects were observed, Dunnett's test was used for multiple comparisons. A P value of < 0.05 was considered significant.

Results

The basal values of $[Ca^{2+}]_i$ were not significantly different between chicken osteoclast precursor cells (day 2–3) (137 ± 10 nM, mean ± SE, n = 43) and multinucleated osteoclasts (day 5–9) (143 ± 13 nM, mean ± SE, n = 20). Replacing the bathing media with KRH containing ipriflavone (10^{-6} M) caused a slow and sustained increase of $[Ca^{2+}]_i$ in chicken osteoclast precursor cells (Fig. 1A). The ipriflavone-stimulated $[Ca^{2+}]_i$ increase remained elevated for at least 10 min. After the addition of the nonspecific Ca^{2+} influx blocker $LaCl_3$ (0.5 mM), $[Ca^{2+}]_i$ returned to basal level (Fig. 1A). Also, under nominally Ca-free conditions (after replacing the medium with Ca-free containing 4 mM EGTA KRH), ipriflavone did not affect $[Ca^{2+}]_i$ (Fig. 1B). These data suggested that the influx of extracellular Ca^{2+} was responsible for the ipriflavone-induced change in $[Ca^{2+}]_i$. The responses of $[Ca^{2+}]_i$ to ipriflavone were the same in differentiated chicken osteoclasts (data not shown), isolated rabbit osteoclasts (Fig. 1C), and mouse osteoclasts in mixed culture (data not shown), although especially in some rabbit osteoclasts, the sustained Ca increases were occasionally accompanied by transient Ca increases (Fig. 1D). We tested the mechanism of the iprifla-

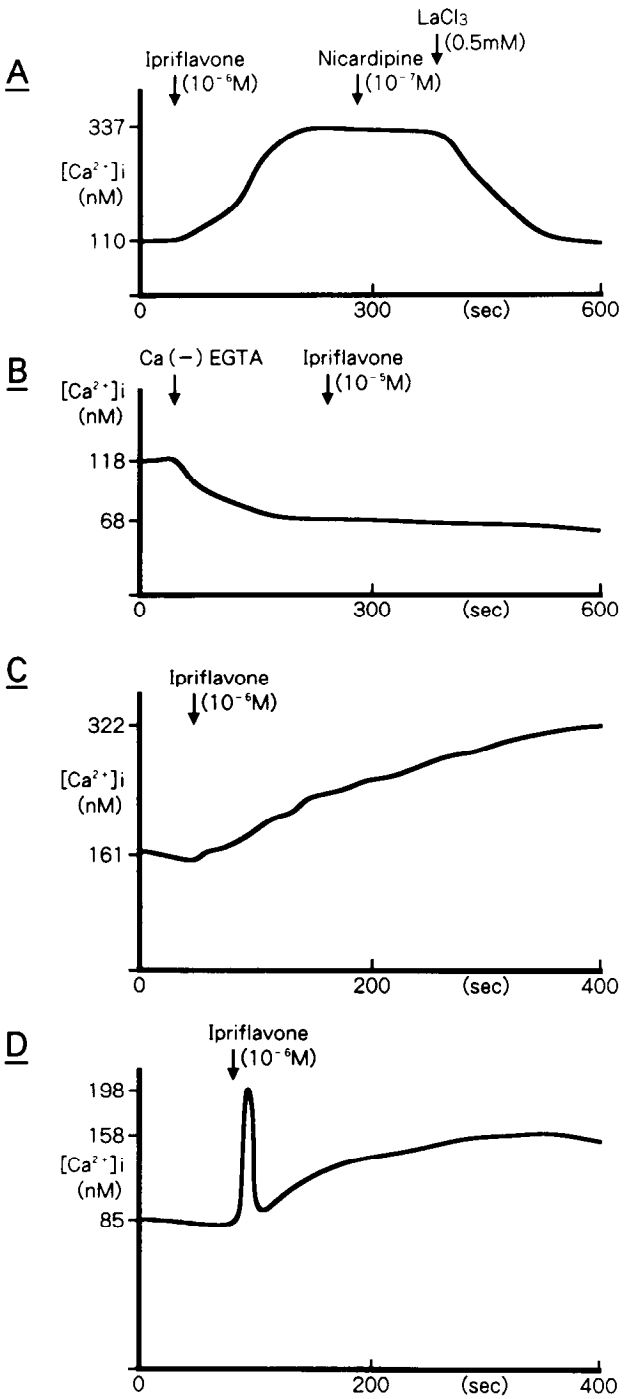

FIG. 1. Effect of ipriflavone on $[Ca^{2+}]_i$ of single chicken osteoclast precursors (A and B) and single rabbit osteoclasts (C and D). Osteoclast precursors or rabbit osteoclasts were grown on coverslips for 1 day. Cells were then loaded with fura-2 and $[Ca^{2+}]_i$ of single cells was measured as described in *Materials and Methods*. A, Increase in $[Ca^{2+}]_i$ in a chicken osteoclast precursor exposed to 10^{-6} M ipriflavone. Lanthanum chloride but not nicardipine prevented this increase. Ipriflavone concentrations were kept constant during the addition of these substances. B, Ipriflavone (10^{-5} M) did not influence $[Ca^{2+}]_i$ of an osteoclast precursor under nominally Ca-free conditions with 4 mM EGTA. C and D, Effect of ipriflavone on $[Ca^{2+}]_i$ of single rabbit osteoclasts. C, Ipriflavone stimulated slow and sustained $[Ca^{2+}]_i$ increases. D, In some cells, ipriflavone induced a rapid and transient $[Ca^{2+}]_i$ increase, followed by a sustained $[Ca^{2+}]_i$ increase.

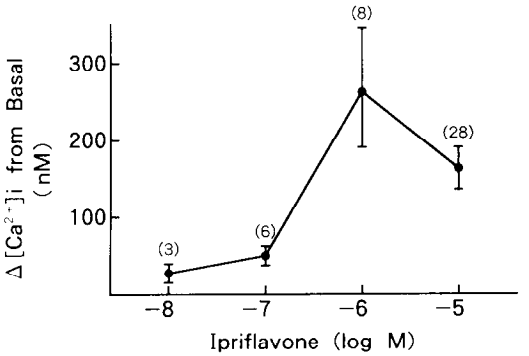

FIG. 2. Dose-response relationship of ipriflavone-induced $[Ca^{2+}]_i$ increase from baseline in chicken osteoclast precursors. Number of cells from several sets of experiments are shown in *parentheses*. Increases in cytosolic calcium measured at 10^{-7} M and higher were significant ($P < 0.05$).

vone-induced Ca^{2+} increase using antagonists of dihydropyridine-sensitive Ca^{2+} channels, nicardipine (10^{-7} M), and verapamil(10^{-7} M). Nicardipine (Fig. 1A) and verapamil (not shown) had no effect on the ipriflavone-induced Ca^{2+} change, suggesting that this increase was derived from dihydropyridine-insensitive Ca influx mechanisms. As shown in Fig. 2, the ipriflavone effect on $[Ca^{2+}]_i$ in chicken osteoclast precursor cells was dose dependent with an EC_{50} of 5×10^{-7} M. The dose response was similar in multinucleated osteoclast-like cells (not shown). The ipriflavone-induced Ca influx was also demonstrated by imaging subcellular Ca^{2+} localization. Chicken osteoclast precursor cells exhibited relatively homogeneous subcellular $[Ca^{2+}]_i$ localization (Fig. 3, *upper*). Three minutes after adding ipriflavone (10^{-5} M), higher submembrane Ca^{2+} values (yellow to red) appeared in most of cells (Fig. 3, *lower*), supporting the notion that ipriflavone stimulated Ca^{2+} influx. In rat osteoclasts, calcitonin induced a Ca influx at least in part through cellular cAMP, the level of which was increased by this hormone (23). We therefore tested whether or not ipriflavone induces changes in cellular cAMP production. As shown in Fig. 4, ipriflavone did not significantly change cellular cAMP production compared with the effect of PGE_2 (11), suggesting that the ipriflavone-induced Ca influx was not through a cAMP pathway. Taken together, the $[Ca^{2+}]_i$ data described above suggests that ipriflavone directly activates a Ca influx pathway.

We then analyzed whether or not ipriflavone binds specific sites in osteoclast-like cells and their precursors. [^{3}H]Ipriflavone binding to osteoclast-like cells was saturable (Fig. 5A). A Scatchard analysis indicated two classes of specific ipriflavone binding sites with dissociation constants (K_d) of 4.98×10^{-8} M and 3.70×10^{-6} M (Fig. 5B). Nonspecific binding comprised approximately 30% of the total binding. Similar binding profiles were observed in precursor cells (K_d, 7.60×10^{-8} M and 2.67×10^{-6} M). As shown in Table 1, specific ipriflavone binding was not influenced by substances that may modulate osteoclast function and $[Ca^{2+}]_i$, such as β-estradiol (24, 25), retinoic acid (26, 27), the voltage-operated Ca channel (VOCC) agonist Bay K8644, the VOCC antagonist nicardipine (8), or Ni and ryanodine, which are potential osteoclast Ca receptor agonists (28). These findings

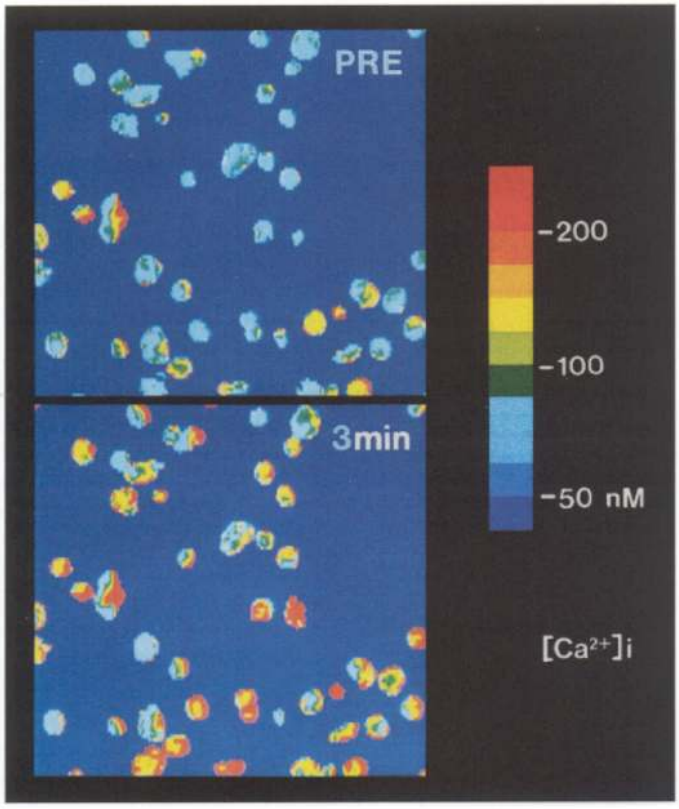

FIG. 3. Digital image analysis of $[Ca^{2+}]_i$ in single fura-2-loaded chicken osteoclast precursors during exposure to ipriflavone (10^{-5} M). Upper, the 340/360 nm ratio image under resting conditions. Lower, $[Ca^{2+}]_i$ distribution in the same cells 3 min after ipriflavone stimulation. The ratio color scale, converted to $[Ca^{2+}]_i$ as described in *Materials and Methods*, is shown on the *right*. As shown in both panels, a total of 50 cells were analyzed. Mean of baselines of the averaged pixel value within each region of interest covering individual whole cell area (20 × 20 pixels) was 101 ± 9 nM (mean ± SE, n = 50). Three minutes after the addition of ipriflavone (10^{-5} M), the mean values increased to 321 ± 44 nM. Response was considered to have occurred when at least a 20 nM $[Ca^{2+}]_i$ increase of averaged pixel values from basal was detected within 3 min; 37 out of 50 cells (74%) responded in this particular experiment. In a total of five sets of experiments, the response rate was 64 ± 14% (mean ± SE, n = 5 experiments, total 162 cells).

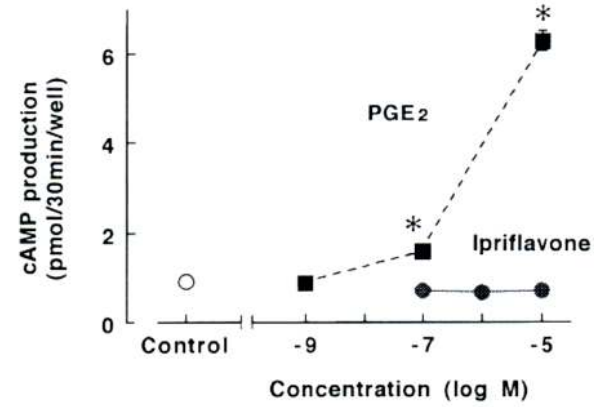

FIG. 4. Effect of ipriflavone on cAMP production in chicken osteoclasts. Multinucleated chicken osteoclast-like cells at day 6 were exposed for 30 min to PGE_2 (10^{-9} M to 10^{-5} M), or ipriflavone (10^{-7} M to 10^{-5} M), and cellular cAMP levels were measured. Values are mean ± SE (n = 6). *, $P < 0.01$ *vs.* control.

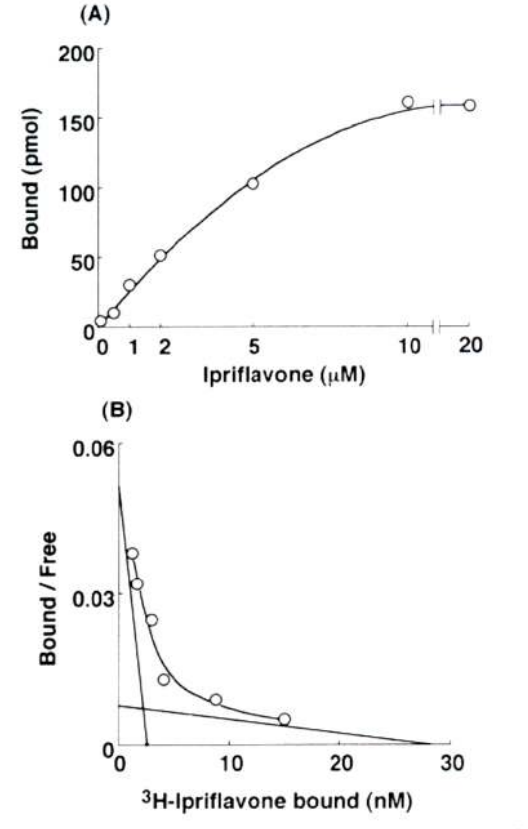

FIG. 5. Saturation curve (A) and Scatchard analysis (B) of [^{3}H]ipriflavone binding to chicken osteoclasts. Multinucleated chicken osteoclast-like cells at day 6 were incubated at 37 C for 3 h with 10^{-8} M [^{3}H]ipriflavone and various concentrations of unlabeled ipriflavone as described in *Materials and Methods*. Each *point* represents mean of six replicates. Comparable results were obtained in three additional experiments.

would suggest that specific ipriflavone binding sites are not identical to the receptors for these substances, VOCCs, or the so-called Ca sensor. Other flavonoids (quercetin and genistein) did not affect the specific binding of ipriflavone (not shown). The data described above could not rule out the possibility that we observed cellular uptake rather than membrane binding of [^{3}H]ipriflavone. We therefore examined [^{3}H]ipriflavone binding using fractionated cellular components, and preliminary data indicated that [^{3}H]ipriflavone binding was specific in membrane fractions (data not shown).

To study the effects of ipriflavone on osteoclast differentiation and activity, we assessed bone resorption by the [^{3}H]proline-labeled bone particle assay (11, 14) and by measuring cellular TRAP activities. TRAP activities did not differ significantly on day 3, however on day 6, TRAP activity was significantly reduced in the ipriflavone-treated groups (10^{-6}M to 10^{-5}M) (Table 2). The bone resorption in the ipriflavone-treated groups (10^{-6}M to 10^{-5} M) was dose dependently reduced between days 2 and 3 in culture when the cells were actively fusing (Fig. 6), suggesting that the ipriflavone effects on cell maturation and fusion inhibited resorption at this stage.

TABLE 1. Effect of various modulators of osteoclast function and $[Ca^{2+}]_i$ on [^{3}H]ipriflavone binding to chicken osteoclasts[a]

Exp 1	
Control	100.0 ± 4.2
Ipriflavone (10^{-5} M)	47.7 ± 1.9[b]
Estradiol (10^{-6} M)	100.8 ± 2.9
Tamoxifen (10^{-6} M)	91.4 ± 3.0
Exp 2	
Control	100.0 ± 1.9
Ipriflavone (10^{-5} M)	54.5 ± 2.1[b]
$NiCl_2$ (5 mM)	97.8 ± 3.7
Nicardipine (10^{-7} M)	89.4 ± 4.6
Bay K8644 (5×10^{-6} M)	88.6 ± 3.5
Exp 3	
Control	100.0 ± 10.6
Ipriflavone (10^{-5} M)	56.7 ± 2.2[b]
Retinoic acid (10^{-6} M)	95.0 ± 6.7
Ryanodine (10^{-4} M)	98.7 ± 5.7

[a] Multinucleated chicken osteoclast-like cells at day 6 were incubated at 37 C for 3 h with [^{3}H]ipriflavone and various calcium transport modulators as described in *Materials and Methods*. Data are expressed as percentage of total control binding of five replicates (mean ± SE) in three sets of experiments.
[b] $P < 0.01$ *vs.* control.

TABLE 2. TRAP activities in control cells and those cultured in the presence of ipriflavone[a]

	nmol(p-nitrophenol)/min/well
Day 3	
Control	17.7 ± 2.0
Ipriflavone (10^{-6} M)	19.1 ± 1.1
Ipriflavone (10^{-5} M)	16.9 ± 0.9
Day 6	
Control	28.9 ± 1.4
Ipriflavone (10^{-6} M)	23.5 ± 1.3[b]
Ipriflavone (10^{-5} M)	18.7 ± 1.2[c]

[a] Chicken osteoclast precursors were cultured in the presence of ipriflavone (10^{-6} M to 10^{-5} M) or control vehicle (ethanol, 0.1%) from day 1. Cellular TRAP activities in each group were measured on day 3 and 6 as described in *Materials and Methods*. Data are presented as mean ± SE of four replicates.
[b] $P < 0.05$.
[c] $P < 0.01$ *vs.* control.

Discussion

We found that ipriflavone directly stimulates $[Ca^{2+}]_i$ in chicken osteoclast precursor cells as well as in mature osteoclasts. The ipriflavone action on $[Ca^{2+}]_i$ is not species-specific, because it affected $[Ca^{2+}]_i$ also in isolated rabbit osteoclasts as well as in rat osteoclasts (29). The ipriflavone effect on Ca release from intracellular stores cannot be totally neglected, because in rabbit cells, the Ca increase was occasionally transient (Fig. 1D). Nonetheless, in chicken precursors and mature osteoclasts, Ca influx from extracellular cell space is the main pathway of increased $[Ca^{2+}]_i$. Ca influx through the cell membrane was observed using a single-cell Ca imaging system as shown in Fig. 3. This is the first report of imaging of the subcellular Ca distribution of osteoclast precursor cells.

Osteoclast activity is very sensitive to changes in $[Ca^{2+}]_i$. Calcitonin stimulates $[Ca^{2+}]_i$ increases possibly by activating phospholipase C as well as Ca influx (10, 23, 30), and it inhibits cell motility and resorptive activity. Stimulation of $[Ca^{2+}]_i$ increase by the Ca ionophore A23187, which stimulates Ca influx across the cell membrane and intracellular Ca release, inhibits osteoclastic bone resorption (10). Increase in $[Ca^{2+}]_i$ by high extracellular Ca^{2+} or Ca^{2+} influx by stimulating VOCC in osteoclasts inhibits their attachment and resorbing activity (8). Ca Influx in osteoclasts is related to the inhibition of osteoclastic bone resorption in the presence of interleukin-4 (31), and the parathyroid hormone-related peptide fragment, osteostatin (32). Although these two factors may act at least in part through VOCC, the ipriflavone-induced Ca influx does not, because the VOCC antagonists nicardipine and verapamil had no effect on ipriflavone-induced Ca changes. Thus, decreased osteoclastic activity is related to Ca influx through either VOCC or other ipriflavone specific Ca influx mechanisms.

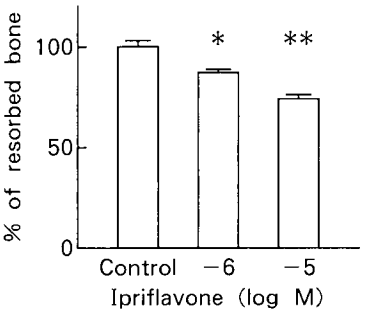

FIG. 6. Bone resorption by control cells and those cultured with ipriflavone from 1–3 days. Bone resorptive activities in each group of cells were assessed by bone particle assay as described in *Materials and Methods*. Data are presented as mean ± SE of four replicates. *, $P < 0.05$; **, $P < 0.01$ *vs.* control.

The role of $[Ca^{2+}]_i$ in the differentiation of osteoclasts remains unknown. In this study, we found that ipriflavone increases $[Ca^{2+}]_i$ in precursor cells and inhibits osteoclast maturation. These findings may suggest that high $[Ca^{2+}]_i$ in precursor cells may be disadvantageous for cell fusion and maturation. However the role of $[Ca^{2+}]_i$ in osteoclast differentiation needs to be further clarified.

Using the chicken osteoclast differentiation model, we found that ipriflavone has two classes of binding sites in osteoclasts and their precursors. This indicates that ipriflavone acts directly on osteoclast lineage cells. Similar ipriflavone binding has been identified in a human leukemia cell line (33), which has some characteristics of osteoclast precursors. The specific binding found in human leukemia cell lines has a single class of sites that differs from those of the native precursor cells. The differences of binding characteristics between these two cell types remain to be clarified.

We studied the signaling systems after ipriflavone binding to two classes of binding sites in native osteoclasts and their precursors. Specific ipriflavone binding was not influenced by substances that may modulate osteoclast function and $[Ca^{2+}]_i$ in our chicken cell model system, such as β-estradiol, retinoic acid, the VOCC agonist and antagonists, or the possible osteoclast Ca receptor agonists. These findings suggest that the specific ipriflavone binding sites are not identical to the receptors for these substances, the VOCC, or the speculated Ca sensors. Preliminary data suggested that osteoblast cell lines (MC3T3-E1) also have specific ipriflavone binding sites. However, ipriflavone does not affect $[Ca^{2+}]_i$ in MC3T3-E1 cells, indicating that ipriflavone binding sites on

osteoblasts do not couple Ca channels. In addition, tamoxifen displaces ipriflavone binding to MC3T3-E1 osteoblast like cells, although this phenomenon was not evident in ipriflavone binding of osteoclasts (Notoya *et al.*, manuscript in preparation). It would be useful to determine how many subtypes of ipriflavone receptors exist.

We have found that ipriflavone (10^{-6} M to 10^{-5} M) inhibited cell differentiation and activity of chicken osteoclast precursor cells, which was reflected by decreased TRAP activities and inhibited bone resorption. The decrease of TRAP activity was dose dependent, particularly at culture day 6; inhibition of bone resorption preceded this process by 3 days. These findings support the reports describing other cell systems (3, 4) in which ipriflavone inhibits osteoclast maturation.

The ipriflavone receptor may be one of the key receptors in osteoclast differentiation. The ipriflavone signaling system may involve the modulation of key factors for osteoclast differentiation, such as the oncogene *c*-src (34), or *c*-fos (14, 35, 36), which stimulates cell differentiation in the chicken cell model used in this study (14). Further studies are necessary to clarify the effects of ipriflavone on bone remodeling at the molecular level.

There are several differences of avian and mammalian osteoclasts in calcium metabolism. The main difference is weak responsiveness to calcitonin (11). However, we think chicken osteoclasts could be used as a model to elucidate ipriflavone action. We have studied $[Ca^{2+}]_i$ measurements and found chicken, rabbit, and rat (29) cells to respond to ipriflavone with $[Ca^{2+}]_i$ increase in an essentially identical manner, suggesting that ipriflavone's action on osteoclasts is universal between species. The reason we have focused mainly on the chicken system is that we are able to obtain a sufficient number of highly purified mature osteoclasts and mononuclear precursor cells for receptor binding studies.

The *in vitro* concentrations of ipriflavone used in this study correspond to plasma concentrations reached when it is orally administered at therapeutic doses (37). The *in vitro* changes in osteoclasts that were observed in the present study may therefore occur in bone tissue after *in vivo* administration of ipriflavone. In fact, oral administration of ipriflavone (38) inhibits the increase in bone resorption associated with local increased numbers of osteoclasts that is induced by the implantation of the parathyroids into rat calvaria.

In summary, we found ipriflavone specific binding, which is coupled to Ca^{2+} influx, in osteoclast precursors and in mature osteoclasts. Ipriflavone directly acts on osteoclast precursors to inhibit maturation and activity, as demonstrated by the inhibition of TRAP and resorptive activity.

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