Transduction of Cell Survival Signals by Connexin-43 Hemichannels*

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Bisphosphonates, drugs used widely in the treatment of bone diseases, prevent osteoblast and osteocyte apoptosis by a mechanism involving extracellular signal-regulated kinase (ERK) activation. We report herein that hexameric connexin (Cx)-43 hemichannels, but not gap junctions, are the essential transducers of the ERK-activating/anti-apoptotic effects of bisphosphonates. Transfection of Cx-43, but not other Cxs, into Cx-43 naïve cells confers de novo responsiveness to the drugs. The signal-transducing property of Cx-43 requires the pore forming as well as the C-terminal domains of the protein, the activation of both Src and ERK kinases, and the SH2 and SH3 domains of Src. This evidence adds Cx-43 to the list of transmembrane proteins capable of transducing survival signals in response to extracellular cues and raises the possibility that it may serve in this capacity for endogenously produced molecules or even other drugs.

Osteocytes, former osteoblasts entombed in the mineralized bone matrix, communicate with each other and with osteoblasts on the bone surface through gap junctions, clusters of channels that connect directly the cytoplasm of neighboring cells (1, 2). Osteocyte death seems to compromise the mechanosensory functions of the osteocyte network and to increase bone fragility (3). Consistent with this notion, the prevalence of osteocyte apoptosis is augmented in conditions associated with increased risk of bone fractures, such as glucocorticoid-induced osteoporosis and sex steroid deficiency (4–6). Conversely, bisphosphonates, stable analogs of pyrophosphate commonly used for the management of osteoporosis, prevent osteocyte and osteoblast apoptosis induced by glucocorticoid excess in vitro and in vivo (7). The anti-apoptotic effect of these agents requires the activation of the extracellular signal-regulated kinases (ERKs). However, the events triggered by bisphosphonates upstream of ERKs have remained heretofore unknown.

Gap junction channels are formed by two hemichannels, each formed by six molecules of connexin (Cx) and contributed by one of the two adjacent cells. The Cx superfamily comprises at least 15 proteins expressed in a tissue-specific manner. Cxs are highly homologous and share similar topology: four membrane spanning domains, two extracellular and one intracellular loop (8). Despite these similarities, channels formed by different Cxs differ in their permeability to intracellular metabolites and second messengers (9, 10). Cx-43 is the major gap junction protein expressed in cells of the osteoblastic lineage and may mediate communication among cells of the osteocyte network (11–13). Lack of Cx-43 in vitro or in vivo results in osteoblast dysfunction and delayed ossification (14, 15).

An essential role of Cxs in physiology has been established by the evidence that spontaneous Cx mutations in humans or loss of Cx function by gene targeting in mice is associated with severe disorders, including demyelinating neuropathies, deafness, congenital cataracts, and even death (16, 17). However, in most cases, knowledge of the direct cause-effect relationship between altered intercellular communication and the defect is lacking. Importantly, different mutations of the same Cx gene cause different disorders (17), suggesting that different domains of the same protein are involved in distinct physiologic processes. Hence, it remains unclear whether the pathologies induced by the specific mutations or by Cx deficiency result from altered gap junction communication or from loss of Cx functions that are independent of gap junctions. A role of Cxs in processes independent of gap junctions has been strongly suggested by evidence that Cx hemichannels operate in nonjunctional regions of the plasma membrane (18–21). In addition, Cx-43 interacts directly with Src, a protein that participates in the transduction of signals induced by a multitude of extracellular stimuli (22, 23). This evidence has raised the possibility that Cx-43 hemichannels may, directly or indirectly, activate intracellular signaling. Thus, Cxs in general, and Cx-43 in particular, may be involved in the communication not just between adjacent cells but also between cells and their extracellular environment.

The studies reported herein demonstrate that Cx-43 hemichannels are essential for the transduction of the anti-apoptotic signal of bisphosphonates and that hemichannel opening leads to the activation of the Src/ERKs signal transduction pathway. These results establish a novel paradigm of Cx-43 function as transducer of extracellular signals and a role for this protein, independent of its well established role in cell-to-cell communication via gap junctions, in the regulation of cell survival.

EXPERIMENTAL PROCEDURES

DNA Constructs and Transient Transfections—Rat Cx-43 and chick Cx-45 cDNAs were provided by Drs. R. Civitelli and T. H. Steinberg (Washington University School of Medicine, St. Louis, MO) (14). The cDNAs for Cx-43Δ245, Cx-43 C-terminal tail, and quadruple Cx-43

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1 The abbreviations used are: ERK(α), extracellular signal-regulated kinase(s); Cx, connexin; nGFP, nuclear green fluorescent protein; FACs, fluorescence-activated cell sorter; LY, lucifer yellow; AGA, 18s-glycerorhytic acid; GA, glycyrrhizic acid; ANOVA, analysis of variance; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine.

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FIG. 1. ERK-mediated survival signals triggered by alendronate require the integrity of Cx channels, but not cell-to-cell contact. Panel A, MLO-Y4 cells were treated for 30 min with vehicle or 100 μM AGA or 100 μM GA followed by the addition of 10^{-7} M alendronate (A) or 10^{-7} M 17β-estradiol (E_2) for 30 min. Subsequently, 10^{-6} M dexamethasone was added, and the percentage of apoptotic cells was quantified after 6 h by trypan blue uptake. Bars indicate the means ± S.D. of triplicate determinations. * p < 0.05 for alendronate- or E_2-treated cultures versus vehicle-treated cultures, by one-way ANOVA. Panel B, cells were treated for 20 min with vehicle, AGA, or GA in serum-free medium, followed by 2 min with 10^{-7} M alendronate or for 5 min with 10^{-7} M 17β-estradiol. ERK phosphorylation was analyzed by Western blotting. Bars indicate fold activation over the respective controls. Results are representative of four independent experiments. Panel C, cells were treated for 20 min with vehicle, carbenoxolone, or oleamide in serum-free medium, followed by 2 min with alendronate. ERK phosphorylation was analyzed by Western blotting. Panel D, MLO-nGFP cells plated at very low density were treated with the indicated agents, as in panel A. The percentage of apoptotic cells was determined by nuclear morphology under an epifluorescent microscope. * p < 0.05 for alendronate-treated cultures versus vehicle-treated cultures, by one-way ANOVA. Panel E, MLO-Y4 cells maintained in suspension were treated for 30 min with vehicle, AGA, GA, or 50 μM PD98059, followed by the addition of alendronate for 30 min. Subsequently, 10^{-6} M dexamethasone was added, and the percentage of apoptotic cells was quantified after 3 h, as in panel A. Panels F and G, cells maintained in suspension were treated with alendronate for 2, 5, or 10 min (panel F) or for 28 min with AGA, GA, or PD98059 followed by 2 min with alendronate (A) (panel G). ERK phosphorylation was analyzed by Western blotting. Results are representative of two and three independent experiments, respectively.
FIG. 2. Functional Cx-43 hemichannels are expressed in osteocytic cells and open upon exposure to alendronate. Panel A, MLO-Y4 cells loaded with calcein-AM (donor cells) were added to monolayers of PKH26-loaded cells (acceptor cells) and treated with vehicle, AGA, or GA for 6 h. Dye transfer from donor to acceptor cells was quantified by enumerating double labeled cells under a confocal fluorescence microscope or by FACS analysis. Panel B, MLO-Y4 cell monolayers were incubated in medium containing 5 mM EGTA or 10−7 M alendronate for the indicated times. LY was added for the last 1 min of incubation. The percentage of fluorescent cells was determined as described under “Experimental Procedures.” * < 0.001 by Wald chi-square. Fluorescence and Nomarski images of the same fields are shown. Panel C, MLO-Y4 cells maintained in suspension were incubated for 15 min with vehicle, AGA, GA, carbeneoxolone, or oleamide. Subsequently, 10−7 M alendronate was added, and cells were incubated for another 15 min. LY was added for the last 1 min of incubation and the uptake quantified as in panel B. Bars indicate the means ± S.D. of triplicate determinations. * p < 0.05 for alendronate-treated cultures versus vehicle-treated cultures, by one-way ANOVA. Panel D, cell surface biotinylation was performed using sulfo-NHS-biotin followed by immunoprecipitation and Western blotting as described under “Experimental Procedures.” n.i., nonimmune IgG.

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Cells—Osteocytic MLO-Y4 cells, MLO-Y4 cells stably transduced with green fluorescent protein targeted to the nucleus (nGFP) named MLO-nGFP, HeLa cells, and embryonic fibroblasts derived from Src-, Yes-, and Fyn-deficient mice (SYP cells) were cultured as described previously (6, 7, 28). ROS17/2.8, ROS17/2.8 stably transfected with Cx-43 were provided by Dr. P. Schwartzberg (National Institutes of Health) (26). SrcΔSH2 and SrcΔSH3 cDNAs were provided by Dr. W. Horne (Yale University School of Medicine, New Haven) (27). All of the constructs used in this study have been shown to produce functional proteins. Cells were transiently transfected using LipofectAMINE (Invitrogen) as described previously (6).

Experimental Procedures. Osteocytic MLO-Y4 cells, MLO-Y4 cells stably transduced with Cx-43, UMR106, and UMR106 transfected with Cx-43 were provided by Dr. T. H. Steinberg and maintained as described previously (14). Embryonic fibroblasts isolated from wild type (Cx-43+/+), Cx-43−/−, Cx-43ΔSH2−/−, and Cx-43ΔSH3−/− cells transfected with Cx-43 were provided by Dr. A. F. Lau (University of Hawaii at Manoa, HI) and cultured as described previously (29). Osteoblastic cells were obtained from neonatal calvaria of wild type or Cx-43-deficient mice (provided by Dr. R. Civitelli) and cultured as described previously (7, 15). The plating density for the different cell types was chosen as to achieve 75–80% confluence after overnight culture. For the experiments evaluating the anti-apoptotic effect of alendronate on very low density adherent cells (see Fig. 1D), MLO-nGFP cells were plated at one-fifth of the normal density. Only single cells, which predominated under these conditions, were examined. For the experiments using cells in suspension, cells were trypsinized, washed with growth medium, and resuspended either in serum-free medium for the experiments in which ERK activation or dye uptake was measured, or in growth medium for the experiments in which apoptosis was evaluated. Subsequently, cells were maintained in suspension using an oscillator operating at 200 rpm placed in a cell culture incubator (20).

Quantification of Apoptotic Cells—Apoptotic cells were quantified either by trypan blue uptake or by evaluating the nuclear morphology of 250–500 cells transfected with nGFP, as described previously (6, 7). Cells exhibiting chromatin condensation and/or nuclear fragmentation were considered apoptotic. Data are presented as a percentage of dexamethasone- or etoposide-induced apoptosis in the absence of alendronate. The percentage of apoptosis was calculated using the formula (% DCp/Dc) × 100, where DCp = dead cells, p = cultures treated with pro-apoptotic agent(s), a = alendronate-treated cultures, and v = vehicle-treated cultures, as published previously (6).

Western Blot Analysis—Phosphorylated ERKs, total ERKs, Cx-43, and β-actin were detected using a mouse monoclonal anti-phosphorylated ERK1/2, a rabbit polyclonal anti-total ERK1/2 (Santa Cruz), a rabbit polyclonal anti-Cx-43 (Zymed Laboratories Inc., San Francisco), or a mouse monoclonal anti-β-actin (Sigma) antibodies, respectively.
Blots were developed by ECL, and the intensity of the bands in the autoradiograms was quantified by scanning and densitometry.

Calcein Transfer and Lucifer Yellow Uptake—Gap junction communication was assessed by the "parachute technique," as described previously (14). Donor cells loaded with calcein-AM (Molecular Probes, Eugene, OR) were added on monolayers of acceptor cells loaded with the permanent membrane red dye PKH26 (Sigma). The number of acceptor cells that received calcein after 6 h was determined by fluorescence confocal microscopy or FACS analysis. The uptake of lucifer yellow (LY) was examined in adherent MLO-Y4 cells plated at half the normal density (to minimize cell-to-cell dye transfer) or in cells maintained in suspension, as described previously (18). The anionic dye rhodamine-dextran with a molecular weight of 10,000 (Molecular Probes, Eugene, OR) was used as a negative control. Cells maintained in serum-free medium containing 1.8 mM Ca were exposed to vehicle, 5 mM EGTA, or 10⁻⁷ M alendronate, in the absence or presence of 18α-glycyrrhetinic acid (AGA), glycyrhizic acid (GA), carbenoxolone, or oleamide, for 1–30 min at 37 °C. 1.25 mg/ml LY (Sigma) was added for the last 1 min of incubation. Cells were washed with the same calcium-containing medium to close the hemichannels, cytospun in the case of cells maintained in suspension, and fixed with neutral buffer formalin. The same fields were observed under epifluorescence or Nomarski microscopy. At least 500 cells from fields selected by systematic random sampling were examined for each experimental condition. Dye uptake was expressed as percentage of fluorescent cells.

Cell Surface Biotinylation—Cell surface biotinylation was performed using sulfo-NHS-biotin as described previously (30) followed by immunoprecipitation with a rabbit anti-Cx-43 antibody or with rabbit non-

Fig. 3. The anti-apoptotic effect of alendronate depends on Cx-43 expression in mouse embryonic fibroblasts and osteoblastic cells. Panel A, Cx-43 expression in the different cell types was examined by Western blotting. Lysates from MLO-Y4 cells, HeLa cells, mouse embryonic fibroblasts from wild type (Cx-43+/+) or Cx-43-deficient (Cx-43−/−) mice, or mouse embryonic fibroblasts from Cx-43−/− or Cx-43+/− mice stably transfected with Cx-43 (Cx-43−/−/43), wild type ROS17/2.8 (ROS) osteoblastic cells, ROS cells stably transfected with Cx-45 (ROS-45), wild type UMR106 (UMR) osteoblastic cells, UMR cells stably transfected with Cx-43 (UMR-43), and authentic osteoblastic cells derived from calvaria of Cx-43−/− or Cx-43−/− mice were used. Western blots were performed with anti-Cx-43 antibody and subsequently with anti-β actin antibody. Panels B–D, the effect of alendronate on dexamethasone (dex)- and etoposide (etop)-induced apoptosis was examined in mouse embryonic fibroblasts (panel B), osteoblast-like cell lines (panel C), and authentic osteoblastic cells derived from murine calvaria (panel D). Apoptosis was examined and quantified as in panel A. Bars indicate the means ± S.D. of triplicate determinations. * p < 0.05 for alendronate-treated cultures versus vehicle-treated cultures, by one-way ANOVA. Panel E, dye uptake in cells maintained in suspension in the absence or presence of alendronate was determined as in Fig. 2C and is expressed as the percentage of fluorescent cells in the presence of alendronate minus the percentage of fluorescent cells in the absence of alendronate. Fluorescence and Nomarski images of the same fields are shown. * p < 0.05, by t test.
immune IgG. Immunoprecipitates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were probed with avidin-horseradish peroxidase followed by anti-Cx-43 antibody.

**Statistical Analysis**—Data were analyzed by one-way analysis of variance (ANOVA), and the Student-Newman-Keuls method was used to estimate the level of significance of differences between means. The effect of bisphosphonate treatment and calcium deprivation on dye uptake was analyzed by Wald chi-square with one degree of freedom (31).

**RESULTS**

**ERK-mediated Survival Signals Triggered by Alendronate Require the Integrity of Cx Channels, but Not Cell-to-Cell Contact**—We examined the possibility that bisphosphonate-induced ERK activation is mediated via modulation of Cx channels using MLO-Y4 osteocytic cells. Prevention of dexamethasone-induced apoptosis, and ERK phosphorylation by the bisphosphonate alendronate, were abrogated in cells pretreated with AGA, an agent that disassembles Cx channels (32, 33) (Fig. 1, A and B). On the other hand, AGA did not modify the anti-apoptosis or ERK activation induced by 17β-estradiol, an effect mediated by extranuclear estrogen receptors (6). The inactive analog of AGA, GA, did not affect anti-apoptosis or ERK activation induced by any of the agonists. 100 μM carbenoxolone and 1 μM oleamide, two other agents reported previously to induce closure of gap junction channels (34), also abrogated anti-apoptosis (data not shown) and ERK activation (Fig. 1C) induced by alendronate. We next determined whether anti-apoptosis by alendronate was dependent on Cx channels involved in gap junctions or on nonjunctional hemichannels. Alendronate prevented dexamethasone-induced apoptosis in MLO-Y4 cells plated at very low density or maintained in suspension to prevent cell-to-cell interactions (Fig. 1, D and E). For the former experiment, we used MLO-Y4 cells stably transduced with nGFP, and apoptosis was quantified by nuclear morphology. Alendronate induced ERK phosphorylation in cells maintained in suspension (Fig. 1F) with a time course similar to that induced in adherent cells (7). Furthermore, the specific inhibitor PD98059 and AGA inhibited alendronate-induced anti-apoptosis and ERK phosphorylation in cells in suspension, but GA did not (Fig. 1, E and G). These results indicate that the anti-apoptotic effect of alendronate does not require cell-to-cell contact and strongly suggest the involvement of nonjunctional Cx hemichannels in the alendronate effect.

**Functional Hemichannels Are Expressed in Osteocytic Cells and Open upon Exposure to Alendronate**—MLO-Y4 cells cultured in monolayers establish intercellular communication through gap junctions, as assessed by the parachute assay (14), a method based on dye transfer from calcein-preloaded donor cells (green) to PKH26-preloaded acceptor cells (red) (Fig. 2A). We determined the number of double-labeled cells, representing acceptor cells that have taken calcein via gap junctions, by confocal fluorescent microscopy (yellow because of the overlap

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**Panel A**, fluorescence images show nuclear morphology of vector- or Cx-43-transfected cells. **Panel B**, cells were transfected with Cx-43 or empty vector. **Panel C**, cells were transiently transfected with Cx-43, Cx-45, Cx-43 + Cx-45, or with empty vector. Cx-43 expression was analyzed by Western blot as in Fig. 3A. **Panel D**, cells were transfected with the indicated Cxs or with empty vector. Apoptosis was induced and examined as described above.

**Fig. 4. Cx-43, but not other Cxs, confers responsiveness to alendronate.** HeLa cells were transiently transfected with expression vectors for the indicated Cxs along with an nGFP expression vector. After 16 h, cells were treated with alendronate for 1 h followed by a 6-h treatment with 100 μM etoposide. Apoptosis was determined by evaluating nuclear morphology of transfected (fluorescent) cells as described under “Experimental Procedures.” **Panel A**, fluorescence images show nuclear morphology of vector- or Cx-43-transfected cells. **Panel B**, cells were transfected with Cx-43 or empty vector. Bars indicate the means ± S.D. of three independent measurements. *p < 0.05 for alendronate-treated cultures versus vehicle-treated cultures, by one-way ANOVA. **Panel C**, cells were transiently transfected with Cx-43, Cx-45, Cx-43 + Cx-45, or with empty vector. Cx-43 expression was analyzed by Western blot as in Fig. 3A. **Panel D**, cells were transfected with the indicated Cxs or with empty vector. Apoptosis was induced and examined as described above.
of green and red confocal images) and by FACS analysis. AGA decreased dye transfer significantly, whereas GA did not. These results are consistent with previous reports demonstrating dye transfer among MLO-Y4 cells or between MLO-Y4 cells and osteoblastic cells (13, 35). Treatment with alendronate, however, did not increase dye transfer (not shown).

We next investigated whether MLO-Y4 cells express functional Cx hemichannels by measuring the uptake of LY, a membrane-nonpermeable fluorescent dye that enters the cell by hemichannels (18). LY uptake by both adherent cells (Fig. 2 B) or cells maintained in suspension (Fig. 2 C) increased upon addition of alendronate or upon removal of extracellular Ca$^{2+}$ by the addition of EGTA, an established maneuver that opens Cx hemichannels (18–20, 36). On the other hand, the uptake of rhodamine-dextran, an anionic fluorescent dye with a molecular weight of 10,000, incapable of passing through hemichannels, was not modified by either alendronate or EGTA in adherent MLO-Y4 cells (2.1 ± 2.5% and 1.5 ± 1.0% fluorescent cells, respectively, versus 1.5 ± 0.9% for vehicle-treated cultures). LY uptake induced by alendronate was reduced significantly by AGA, carbonoxolone, or oleamide, but not by GA. On the other hand, alendronate increased LY uptake and prevented apoptosis even in the presence of suramin, a competitive antagonist of P2 purinergic receptors (37) (data not shown). These results, together with the evidence that AGA does not interfere with purinergic receptors (38), exclude the involvement of these receptors in alendronate effect.

We next examined whether Cx-43, the main Cx expressed in MLO-Y4 cells (13, 35), was a component of hemichannels. Cell surface biotinylation was performed using sulfo-NHS-biotin, a membrane-impermeant reagent that biotinylates extracellular domains of transmembrane proteins and has restricted access to Cxs involved in gap junctions (30, 39). After biotinylation, cells were lysed and immunoprecipitated with rabbit anti-Cx-43 antibody but not by the nonimmune IgG. The identity of Cx-43 was confirmed by probing the same membrane with anti-Cx-43 antibody (Fig. 2 D). Taken together, these results indicate that Cx-43 is present in nonjunctional cell membranes and that osteocytic cells express functional Cx-43 hemichannels that open upon exposure to alendronate.

Anti-apoptosis Induced by Alendronate Depends on Cx-43 Expression in Mouse Embryonic Fibroblasts and Osteoblastic Cells—The ability of alendronate to inhibit apoptosis was examined in several cell models that do, or do not, express Cx-43 (Fig. 3 A). Alendronate prevented dexamethasone- or etoposide-induced apoptosis of embryonic fibroblasts derived from wild type or from Cx-43-deficient mice (Cx-43$^{-/-}$) stably transfected with Cx-43 (Cx-43$^{-/-}$/Cx-43). On the other hand, it did not inhibit apoptosis of embryonic fibroblasts derived from Cx-43$^{-/-}$ mice (Fig. 3 B). Moreover, alendronate prevented apoptosis of ROS17/2.8 cells, which express Cx-43. However, it had no effect on UMR106 cells, which express very low levels of Cx-43 (Fig. 3 C). Furthermore, as in the case of the embryonic fibroblasts, alendronate prevented apoptosis of UMR106 cells stably transfected with Cx-43 (UMR43). On the other hand, overexpression of Cx-45 in ROS17/2.8 caused loss of responsiveness to alendronate (Fig. 3 C). This latter phenomenon could be the result of decreased expression of Cx-43 in these cells (Fig. 3 A), or of a “dominant negative-like” effect by which overexpression of Cx-45 reduces Cx-43-mediated responses (14, 40), or of a combination of both. Moreover, authentic osteoblasts derived from calvaria of neonatal homozygous Cx-43$^{-/-}$ mice were refractory to alendronate-induced anti-apoptosis, whereas apoptosis was prevented by alendronate in calvarial osteoblasts derived from wild type (Cx-43$^{-/-}$) mice (Fig. 3 D).

The anti-apoptotic effect of alendronate correlated with induction of LY uptake by this agent in cells maintained in suspension (Fig. 3 E).

Cx-43, but Not Other Cxs, Confers Responsiveness to Alendronate—HeLa cells, which do not express Cx-43 (Figs. 3 A and 4 C), were transfected with an expression vector containing Cx-43 or with the empty vector, along with an expression vector for nGFP to allow for apoptosis quantification only of transfected (fluorescent) cells (Fig. 4 A). Alendronate had no effect on etoposide-induced apoptosis in HeLa cells transfected with empty vector. On the other hand, it abolished apoptosis of cells transfected with Cx-43 (Fig. 4, A and B). Cx-43 protein expression upon transfection with the Cx-43 plasmid was confirmed by Western blot analysis (Fig. 4 C). HeLa cells were also transfected with expression plasmids for Cx-26, Cx-31, Cx-32, Cx-37, Cx-40, or Cx-45 along with nGFP, and apoptosis was quantified as in Fig. 4 A. Alendronate did not prevent apoptosis of cells transfected with any of these Cxs, indicating that Cx-43 is unique among these Cxs in conferring responsiveness to alendronate. Furthermore, cotransfection of Cx-45 abolished anti-apoptosis by alendronate in Cx-43-transfected cells (Fig. 4 D). These results are consistent with the results of Fig. 3 C, in which overexpression of Cx-45 in Cx-43-expressing ROS17/2.8 cells abolished anti-apoptosis by alendronate. In contrast to the findings with ROS-45 cells in which Cx-43 expression is lower than in wild type ROS17/2.8 cells (Fig. 3 A), transfection of Cx-45 together with Cx-43 in HeLa cells did not change Cx-43 expression significantly (Fig. 4 C). These results suggest that Cx-45 inhibits Cx-43-mediated anti-apoptosis by a mechanism independent of the level of Cx-43 expression.

Anti-apoptosis by Alendronate Requires Src Interaction with Cx-43 and Src Kinase-mediated Activation of ERKs—The cytoplasmic C-terminal domains of Cxs differ considerably (41), indicating that the specific requirement of Cx-43 for the anti-apoptotic effect of bisphosphonates could be caused by this region. Consistent with this contention, we found that alendronate did not prevent apoptosis in HeLa cells expressing a truncated form of Cx-43 (Cx-43A245), which lacks the C-terminal domain. On the other hand, coexpression of this mutant together with the Cx-43 C-terminal peptide (C-tail), but not the Cx-43 C-tail alone, did confer alendronate-induced anti-apoptosis (Fig. 5 A).

The kinase Src, an activator of the ERK pathway (28), associates with Cx-43 through SH2 and SH3 binding sites present in the C terminus of Cx-43 (42); and Cx-43 phosphorylation by Src or ERKs induces channel closure (24, 43). Therefore, we hypothesized that opening of Cx-43 hemichannels by alendronate could induce Src activation followed by ERK phosphorylation. In this scenario, Src activation would not only trigger survival signals via ERK activation, but would also close Cx-43 hemichannels and preserve cellular homeostasis. Therefore, we evaluated whether Src expression and/or activity was required for the anti-apoptotic effect of alendronate. We found that anti-apoptosis and ERK activation induced by alendronate in MLO-Y4 osteocytic cells were abolished by the specific inhibitor of Src kinases, PP1 (Fig. 5, B and C). Moreover, and in contrast to its anti-apoptotic effect on wild type embryonic fibroblasts (shown in Fig. 3 B), alendronate did not induce anti-apoptosis or ERK activation in embryonic fibroblasts derived from Src-deficient mice (Src$^{-/-}$) (Fig. 5, D and E). Transfection of Cx-43 to these cells did not confer responsiveness to alendronate, demonstrating that lack of response is caused by the lower expression of Cx-43 in Src$^{-/-}$ embryonic fibroblasts compared with...
FIG. 5. The anti-apoptotic effect of alendronate requires Src interaction with Cx-43 and Src kinase-mediated activation of ERKs. Panel A, HeLa cells were transiently transfected with expression vectors for wild type Cx-43, a Cx-43 mutant lacking the C-terminal tail (Cx43Δ245), the C-terminal peptide (C-tail), or both the Cx-43Δ245 together with the C-terminal peptide, along with an nGFP expression vector. Apoptosis was evaluated as in Fig. 4. Bars indicate the means ± S.D. of three independent measurements. *p < 0.05 for alendronate-treated
wild type cells (Fig. 5, D and F). On the other hand, transfection of Cx-43 and Src, or even Src alone, restored the anti-apoptotic effect of alendronate, confirming that Src expression is required for this action (Fig. 5D). Consistent with these results, alendronate prevented etoposide-induced apoptosis in HeLa cells expressing Cx-43 and wild type Src. However, cells transfected with Cx-43 together with either a dominant negative Src mutant that lacks kinase activity (Src K–) or dominant negative Src mutants lacking the SH2 or SH3 domains, which display defective interaction with Cx-43 (42), were not protected from apoptosis by alendronate (Fig. 5G). These results demonstrate that Src activity is required for alendronate-induced anti-apoptosis and suggest that interaction between Cx-43 and Src is also necessary.

Finally, consistent with the evidence that inhibition of the kinase MEK, which is responsible for ERK activation, abolished alendronate-induced anti-apoptosis in osteocytic cells (Fig. 1 and Ref. 7), cotransfection of a dominant negative MEK along with Cx-43 abolished anti-apoptosis by alendronate (Fig. 5H). Furthermore, a Cx-43 mutant incapable of being phosphorylated by ERKs (Cx-43-4S/A) (24) conferred anti-apoptosis as effectively as wild type Cx-43, indicating that Cx-43 phosphorylation by ERKs is not required for anti-apoptosis.

**DISCUSSION**

The studies reported in this paper demonstrate that the bisphosphonate alendronate, a small synthetic molecule of 250 Da, efficacious in the treatment of several metabolic bone dis-

cases, exerts anti-apoptotic effects on osteocytes and osteo-
blasts by opening Cx-43 hemichannels and that hemichannel opening in turn activates the Src kinase and ERKs. These findings establish a previously unsuspected role of Cx-43 in the regulation of cell survival and elucidate a novel function of this protein, in addition to its well known role in gap junction communication. Moreover, our findings with alendronate imply that naturally occurring small molecules must exist in the extracellular milieu which deliver anti-apoptotic signals through their ability to gate Cx-43 hemichannels.

Heretofore, the only known biologic role of Cxs was in inter-
cellular communication and specifically the formation of full channels (gap junctions) between neighboring cells. Trafficking of small molecules through gap junctions was known to exert regulatory influences on cell proliferation and differentiation. While our manuscript was in preparation, Kamermans et al.
(21) reported that hemichannels formed by Cx-26 are critical for the transmission of signals between neurons in the retina. The evidence presented herein that MAP kinases can be activated by hemichannel opening and that Cxs play a role in the transduction of survival signals in response to extracellular cues adds Cx-43 hemichannels to the large list of transmembrane proteins, e.g. growth factor receptors, G protein coupled receptors, integrins, etc., capable of transducing extracellular signals to intracellular signaling pathways that influence cell survival.

The precise mechanism by which exposure to alendronate leads to opening of Cx-43 hemichannels in osteocytes/osteoblasts is a matter of conjecture at the present time. Osteoclasts, the highly specialized polykaryons responsible for the resorption of bone, like osteoblasts and osteocytes, are targets of bisphosphonate action. Specifically, bisphosphonates act on osteoclasts to induce their apoptosis (44). This mechanism underlies the potent antiresorptive actions of this class of drugs. Some bisphosphonates, including alendronate, trigger osteo-
clast apoptosis by inhibiting enzymes of the mevalonate path-
way which prenylate survival proteins, such as Ras, whereas others form cytotoxic analogs of ATP (45–47). It is thought that bisphosphonates buried in the bone matrix by virtue of their high affinity for hydroxyapatite reach the osteoclast interior following osteoclastic bone resorption and the phagocytosis of particles of matrix containing the drug (48). However, the results of the present report together with evidence that osteoclasts express Cx-43 (49) raise the possibility that both in osteoblastic/osteocytic cells and osteoclasts the drugs might enter the interior of the cells through Cx-43 hemichannels. In any event, the absolute requirement of Cx-43 for the effect of alendronate on osteoblasts and osteocytes, and for that matter in HeLa cells transfected with Cx-43, strongly suggests that bisphosphonates either interact directly with this protein or somehow recognize and interact with another membrane moi-
ety associated with Cx-43. Hemichannel opening may result from direct or indirect interaction of bisphosphonates with Cx-43, which leads to alteration of the tertiary and/or quater-

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cultures versus vehicle-treated cultures, by one-way ANOVA. Panel B, MLO-Y4 cells were treated for 30 min with vehicle or 10 μM PP1 followed by incubation with alendronate for another 30 min. Apoptosis was induced by the addition of 10−7 M dexamethasone or 50 μM etoposide for 6 h and quantified as in Fig. 1A. Panel C, MLO-Y4 cells were treated for 28 min with vehicle or PP1 followed by the addition of alendronate for 2 min. ERK phosphorylation was examined by Western blotting. Panel D, embryonic fibroblasts derived from mice deficient in Src kinases (Src−/−) were transfected with empty vector, Cx-43, wild type Src, or both Cx-43 and Src, along with nGFP. Cells were treated for 1 h with alendronate followed by induction of apoptosis by etoposide as in panel A. Panel E, Src−/− or wild type (Src+/+) mouse embryonic fibroblasts were treated for 2 min with alendronate, and ERK phosphorylation was analyzed by Western blotting. Results are representative of three independent experiments. Panel F, Cx-43 expression in Src−/−, Src−/−, or Src−/− mouse embryonic fibroblasts transiently transfected with a Cx-43 expression plasmid or with the empty vector was analyzed by Western blotting. Panel G, HeLa cells were transiently transfected with an expression vector for Cx-43 alone or in combination with wild type Src, or a kinase-deficient Src (Src K−), or a Src mutant lacking the SH2 domain (SrcΔSH2), or a Src mutant lacking the SH3 domain (SrcΔSH3), along with a nGFP expression vector. Apoptosis was evaluated as in panel A. Panel H, HeLa cells were transiently transfected with expression vectors for Cx-43, a dominant negative MEK alone or in combination with Cx-43, or a Cx-43 mutant in which serines 255, 257, 279, and 292 had been replaced by alanines (Cx-43-4S/A) along with a nGFP expression vector. Apoptosis was evaluated as in panel A.
nary structures of Cx-43. Future studies will of course be needed to establish the nature of this putative “cell-like” role of Cx-43 in alendronate action.

Each of the six Cx-43 proteins making up the hemichannel has two distinct domains: a transmembrane domain that participates in the formation of the pore of the hemichannel and a C-terminal domain that contains the proline-rich SH3 and SH2 binding sites required for the interaction of Cx-43 with Src. The results of our experiments with truncated mutants consisting of only the pore forming domain or the C-terminal domain indicate that both regions are needed for the anti-apoptotic effect of alendronate. In addition, because the truncated form of Cx-43 lacking the C-tail (Cx-43Δ245) forms channels with permeability similar to that exhibited by wild type Cx-43 (24), the inability of this mutant to transduce the anti-apoptotic effect of alendronate implies that communication between the cytoplasm and the extracellular space is not sufficient for this effect. In fact, the restoration of the anti-apoptotic effect of alendronate in HeLa cells by cotransfection of the pore forming region together with the C-terminal tail in our experiments is fully consistent with the contention that the transduction of the cell survival signal requires the association of the two portions of the molecule through intramolecular noncovalent interactions, the so-called “ball-and-chain” mechanism described previously by Hoshi and co-workers (50). Interestingly, the ball-and-chain mechanism has been also evolved to explain the closing of Cx-43 gap junction channels in response to Src, pH changes, as well as insulin (24, 51, 52).

Based on our results and the lines of supporting evidence discussed above, we propose that a direct or indirect interaction of alendronate with the Cx-43 pore forming region is responsible for opening the hemichannel, whereas the Cx-43 C-terminal portion of the molecule serves as the docking site for Src kinase(s) or other adaptor molecules. The subsequent Src activation evidently links Cx-43 with the ERK pathway (Fig. 6). The sequential nature of the events depicted in the figure is fully supported by the results of our experiments with dominant negative kinases (Fig. 5). Our model also provides for the transient nature of the hemichannel opening by bisphosphonates. Specifically, we propose that activation of Src and ERKs not only will attenuate cell death but will also cause the closure of the hemichannels by phosphorylating the C terminus of Cx-43. This particular contention is fully supported by previous evidence that phosphorylation of the C-terminal portion of Cx-43 by these kinases results in channel closure (24, 42, 43). Whether bisphosphonates induce phosphorylation of Cx-43 will require further studies.

At this stage, the identity of putative naturally occurring small molecules that may control Cx-43 hemichannel opening leading to survival signals is only a matter of conjecture. None-theless, changes in calcium concentrations within the physiological range as well as removal of extracellular calcium by chelators, such as EGTA, do open Cx-43 hemichannels (19). Extracellular ATP and metabolic inhibitors also open Cx-43 hemichannels. However, in distinction to extracellular calcium removal or bisphosphonates, extracellular ATP and metabolic inhibitors induce permanent opening of the hemichannels and cell death (18, 53–55).

Although a major action of bisphosphonates is prevention of bone resorption resulting from the drugs’ ability to induce osteoclast apoptosis, bisphosphonates have been surprisingly effective in the management of skeletal disorders unrelated to abnormal osteoclastic resorption. Indeed, treatment with bisphosphonates benefits children with severe forms of osteogenesis imperfecta, an autosomal dominant familial disease caused by abnormalities in the synthesis of collagen type I by osteoblasts (56). Likewise, bisphosphonates seem efficacious in idiopathic juvenile osteoporosis, a disease of decreased osteo-blast performance of unknown etiology (57). On the other hand, targeted disruption of the Cx-43 gene in mice results in delayed ossification and generalized osteoblast dysfunction (15). Taken together with the results of the present report, these observations raise the possibility that Cx-43-transduced survival signals are indeed of critical importance for the normal function of osteoblasts. Consequently, gating of Cx-43 hemichannels by small molecules present in the bone microenvironment may be one important mechanism for maintaining osteoblast survival and thereby skeletal homeostasis.

In conclusion, the findings reported in this paper elucidate a novel paradigm of signal transduction mediated by Cx-43 and open new avenues of investigation aiming to examine the possibility that Cx-43 hemichannels are utilized by endogenous molecules and perhaps drugs other than bisphosphonates to transduce cell survival signals.

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Transduction of Cell Survival Signals by Connexin-43 Hemichannels
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