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Cytosolic Entry of Bisphosphonate Drugs Requires Acidification of Vesicles after Fluid-Phase Endocytosis

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ABSTRACT

Bisphosphonates such as alendronate and zoledronate are blockbuster drugs used to inhibit osteoclast-mediated bone resorption. Although the molecular mechanisms by which bisphosphonates affect osteoclasts are now evident, the exact route by which they are internalized by cells is not known. To clarify this, we synthesized a novel, fluorescently labeled analog of alendronate (AF-ALN). AF-ALN was rapidly internalized into intracellular vesicles in J774 macrophages and rabbit osteoclasts; uptake of AF-ALN or [^{14}C]zoledronate was stimulated by the presence of Ca^{2+} and Sr^{2+} and could be inhibited by addition of EGTA or clodronate, both of which chelate calcium ions. Both EGTA and clodronate also prevented the bisphosphonate-induced inhibition of Rap1A prenylation, an effect that was reversed by addition of Ca^{2+}. In J774 cells and osteoclasts, vesicular AF-ALN colocalized with dextran (but not wheat germ agglutinin or transferrin), and uptake of AF-ALN or [^{14}C]zoledronate was inhibited by dansylcadaverine, indicating that fluid-phase endocytosis is involved in the initial internalization of bisphosphonate into vesicles. Endosomal acidification then seems to be absolutely required for exit of bisphosphonate from vesicles and entry into the cytosol, because monensin and bafilomycin A1, both inhibitors of endosomal acidification, did not inhibit vesicular uptake of AF-ALN or internalization of [^{14}C]zoledronate but prevented the inhibitory effect of alendronate or zoledronate on Rap1A prenylation. Taken together, these results demonstrate that cellular uptake of bisphosphonate drugs requires fluid-phase endocytosis and is enhanced by Ca^{2+} ions, whereas transfer from endocytic vesicles into the cytosol requires endosomal acidification.

Bisphosphonates are nonhydrolyzable analogs of pyrophosphate that inhibit bone resorption and have been used for more than 3 decades in the treatment of Paget's disease of bone and, more recently, for the treatment of tumor-induced osteolysis, postmenopausal osteoporosis, and other metabolic bone diseases (Russell and Rogers, 1999). By virtue of their ability to bind to Ca^{2+} ions, bisphosphonates rapidly localize to bone mineral in vivo and accumulate beneath bone-resorbing osteoclasts (Sato et al., 1991; Azuma et al., 1995; Masarachia et al., 1996) that subsequently release and internalize the bisphosphonates in the acidic environment of the resorption lacuna. Once internalized by osteoclasts, the bisphosphonates have two distinct mechanisms of action depending on the structure of their R_2 side chain (Benford et al., 1999). Nitrogen-containing bisphosphonates (N-BPs), such as alendronate and zoledronic acid (zoledronate) inhibit farnesyl diphosphate synthase, an enzyme in the mevalonate pathway (van Beek et al., 1999; Bergstrom et al., 2000; Dunford et al., 2001). In addition to being required for cholesterol biosynthesis, the mevalonate pathway catalyzes the synthesis of the isoprenoid lipids, farnesyl diphosphate and geranylgeranyl diphosphate, which are the substrates for post-translational lipid modification (prenylation) of small GTPases such as Rap, Rac, Rho, and Cdc42 (Luckman et al., 1998b; Rogers, 2003). Inhibition of farnesyl diphosphate synthase prevents the synthesis of farnesyl diphosphate and geranylgeranyl diphosphate, causing the accumulation of the unprenylated forms of small GTPases, thereby disrupting osteoclast function (Coxon et al., 2001; Rogers, 2003). By contrast, the non–N-BPs, such as clodronate, do not inhibit farnesyl diphosphate synthase (Dunford et al., 2001) but are metabolized intracellularly by osteoclasts to nonhydrolyzable analogs of ATP that induce osteoclast apoptosis (Auriola et al., 1997; Frith et al., 1997, 2001).

Despite the recent major advances in understanding the molecular mechanisms of action of bisphosphonates, the

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ABBREVIATIONS: N-BP, nitrogen-containing bisphosphonate; AF-ALN, fluorescently labeled analog (AlexaFluor-488) of alendronate; PBS, phosphate-buffered saline; MEM, minimum essential medium; TAMRA, tetramethylrhodamine; FITC, fluorescein isothiocyanate; GGTI, geranylgeranyl transferase inhibitor.

mechanism by which they are internalized by osteoclasts during bone resorption is still not understood. We demonstrated recently that the non-N-BP clodronate prevented alendronate-induced apoptosis in J774 macrophage-like cells and prevented the alendronate-induced inhibition of prenylation of the small GTP-binding protein Rap1A in J774 macrophage-like cells and in rabbit osteoclasts (Frith and Rogers, 2003). We suggested that this may be the result of reduced uptake of alendronate, because clodronate also partially prevented the uptake of [^{14}C]-labeled ibandronate. These results suggest that there might be a specific recognition step in the internalization process.

In the present study, we have investigated the mechanism by which bisphosphonates are internalized into J774 macrophage-like cells and compared this to uptake into osteoclasts. We synthesized a novel, fluorescently labeled analog of alendronate (AF-ALN) to visualize and quantify the uptake of alendronate into cells by confocal microscopy and flow cytometry. We then determined whether bisphosphonates or other agents that interfere with endocytosis or vesicular acidification inhibited the cellular uptake of AF-ALN or radiolabeled zoledronate. Because flow cytometric analysis provides an indication of the total (e.g., vesicular *and* cytosolic) amount of AF-ALN within the cells, we also examined the effect of these agents on bisphosphonate-induced accumulation of unprenylated Rap1A in J774 cells, an indication of the entry of N-BPs into the cytosol/peroxisomes and inhibition of farnesyl diphosphate synthase.

Materials and Methods

Reagents. Clodronate and alendronate were a kind gift of Procter and Gamble (Cincinnati, OH) and the hydrated sodium salt of zoledronic acid (zoledronate) and [^{14}C]zoledronate (specific activity, 1.936 GBq/mmol) were kindly provided by Novartis (Basel, Switzerland). Stock solutions of bisphosphonates were prepared in phosphate-buffered saline (PBS), the pH was adjusted to 7.4 with 5 M NaOH and solutions were filter-sterilized before use. Cell culture reagents were from Sigma (Poole, UK). Fluorescently labeled dextran, wheat germ agglutinin, and transferrin were from Invitrogen (Paisley, UK). All other chemicals were purchased from Sigma, unless stated otherwise.

Synthesis of Fluorescently Labeled Alendronate. A quantity of 1.13 μmol of the amine-reactive probe Alexa Fluor-488 carboxylic acid 2,3,5,6-tetrafluorophenyl ester (AF-488; Invitrogen) dissolved in DMSO was mixed with 11.3 μmol of alendronate [dissolved in bicarbonate buffer, pH 9.0 (i.e., a 1:10 molar ratio)]. The volume was made up to 1 ml with distilled water and the solution incubated for 2 h at room temperature with mixing. To precipitate alendronate, 19.8 μmol of $CaCl_2$ were added and the mixture was centrifuged (14,000*g*, 10 min). The precipitate was washed five times in 1 ml of distilled water. To bind the Ca^{2+} (and hence resolubilize the alendronate), 19.8 μmol of EGTA was added to the precipitate. PBS (100 μl) was added until all alendronate-AlexaFluor-488 (AF-ALN) had dissolved and the solution was mixed for 30 min. The final solution (referred to as AF-ALN) contained approximately 7% labeled alendronate and 93% free alendronate.

Cell Culture. J774 macrophage-like cells were cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. For quantification of AF-ALN internalization by flow cytometry, J774 cells were seeded into 24-well tissue culture plates at a density of 2×10^5 cells/well. For the measurement of [^{14}C]zoledronate internalization, J774 cells were seeded into 12-well tissue culture plates at a density of 4×10^5 cells/well. For visualization of AF-ALN uptake by confocal microscopy, cells were seeded onto glass coverslips in 24-well plates at a density of 1×10^5 cells/well. For Western blot analysis, J774 cells were seeded into 12-well plates at a density of 4×10^5 cells/well. In all cases, after 16 h the culture medium was replaced with fresh medium containing test reagents and the cells were cultured for a further 4 to 6 h.

Isolation and Culture of Rabbit Osteoclasts. To visualize uptake of AF-ALN by rabbit osteoclasts, the cells were isolated as described previously (Coxon et al., 2000). In brief, the long bones from neonatal rabbits (3–4 days old) were cleaned and minced in serum-free α-minimal essential medium (α-MEM). The suspension of bone chips was vortexed and, after allowing the bone chips to settle out, the bone cell suspension, was plated onto glass coverslips in 24-well plates. After approximately 16 h in culture [α-MEM containing 10% (v/v) fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mM glutamine], the cells were washed repeatedly with PBS to remove the contaminating stromal cells, and then the osteoclasts were cultured in complete α-MEM.

For Western blot analysis, osteoclast-like cells were generated from rabbit bone marrow using a method modified from David et al. (1998). The bone cell suspension described above was seeded at a density of 5×10^6 cells/well in a six-well plate in complete α-MEM supplemented with 10^{-8} M 1,25-dihydroxyvitamin D_3. The medium was replaced on day 5. When an almost confluent layer of osteoclast-like cells had formed (approximately days 10–14), the cells were washed gently with PBS to remove any remaining stromal cells. These cultures contain >95% pure multinucleated, tartrate-resistant acid phosphatase-positive, osteoclast-like cells (Coxon et al., 2003). The osteoclast-like cells were treated (in complete α-MEM) for 24 h and then lysed for Western blot analysis.

Confocal Microscopy. To determine whether AF-ALN was internalized by adsorptive, receptor-mediated, or fluid-phase endocytosis, cells were incubated with 1 μg/ml wheat germ agglutinin-633 (a marker for adsorptive endocytosis), 20 μg/ml transferrin-633 (a marker for receptor-mediated endocytosis), or 250 μg/ml tetramethylrhodamine-labeled dextran (TAMRA-dextran, a marker of fluid-phase endocytosis) together with 100 μM AF-ALN. To determine whether clodronate itself had an effect on fluid-phase endocytosis, J774 cells or rabbit osteoclasts were treated with 100 μM AF-ALN + 500 μg/ml TAMRA-dextran (M_r 10,000) ± 1 mM clodronate. After treatment for 6 h, the cells were washed in PBS and fixed for 10 min in 4% (v/v) formaldehyde. Cells were examined on a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss Ltd., Welwyn Garden City, UK) and images captured using the LSM image capture software (Carl Zeiss Ltd).

Flow Cytometric Analysis. Flow cytometric analysis was used to quantify the uptake of AF-ALN by J774 cells over 4 h of treatment and to evaluate the effect of various agents on the internalization. To examine the overall accumulation of AF-ALN, J774 cells were treated with 100 μM AF-ALN for 1 to 4 h. For the cells treated for less than 4 h, the AF-ALN was removed at the specified time and replaced with fresh medium. All cells were then harvested 4 h after the start of the experiment.

To determine the effect of divalent ions (Ca^{2+}, Sr^{2+}, and Mg^{2+}) on the uptake of AF-ALN, the cells were treated with 100 μM AF-ALN ± 1 mM $CaCl_2$, 1 mM $SrCl_2$, or 1 mM $MgCl_2$. To further investigate the effect of Ca^{2+} on the uptake of bisphosphonates, we measured AF-ALN uptake in the presence and absence of Ca^{2+} ions. J774 cells were treated with 100 μM AF-ALN alone or together with 100 to 1000 μM EGTA or 100 to 1000 μM clodronate. To confirm that the effect of clodronate and EGTA on AF-ALN uptake was the result of chelation of Ca^{2+} from the medium rather than a direct cellular effect (e.g., toxicity), J774 cells were pretreated with 1 mM clodronate or 1 mM EGTA for 4 h, then cultured for a further 4 h in fresh medium containing 100 μM AF-ALN with either clodronate or EGTA, or in fresh medium containing 100 μM AF-ALN alone. We also examined the effect of clodronate and EGTA on the uptake of AF-ALN in calcium-free DMEM. J774 cells were treated with 100

μM AF-ALN ± 1 mM EGTA, 1 mM clodronate, or 1 mM $CaCl_2$ for 4 h in calcium-free DMEM.

To examine whether endocytosis was involved in the uptake of AF-ALN, cells were treated for 4 h with either 100 μM AF-ALN or 250 μg/ml FITC-dextran ± 500 μM dansylcadaverine (Sigma, Poole, UK) and to examine the requirement for vesicular acidification, cells were treated for 4 h with 100 μM AF-ALN or 250 μg/ml FITC-dextran ± 20 μM monensin, an electroneutral monovalent ionophore (Kaiser et al., 1988) or 50 nM bafilomycin A1, an inhibitor of the vacuolar H^+-ATPase (Bowman et al., 1988). To further clarify the endocytotic process involved in bisphosphonate internalization, J774 cells were incubated for 1 h in the presence of 100 μM AF-ALN, 250 μg/ml FITC-dextran, 10 μg/ml wheat germ agglutinin-633, or 20 μg/ml transferrin-633 at either 4°C or 37°C. The cells were then washed in PBS and incubated in drug-free medium at 37°C for a further 3 h.

In all of the above experiments, cells were harvested, centrifuged (3200*g*, 5 min) and washed three times in PBS. The pellet was then resuspended in 300 μl of 1% (v/v) formaldehyde. The samples were vortexed and analyzed on a FACSCalibur (BD Biosciences) using the 488 nm laser.

[^{14}C]Zoledronate Internalization. J774 cells were seeded into 12-well tissue culture plates and allowed to adhere overnight. The medium was then replaced with fresh medium containing 25 μM [^{14}C]zoledronate (specific activity, 1.936 GBq/mmol) ± other agents for 4 h. Cells were then washed four times in PBS and lysed in 50 mM Tris, pH 7.7, 0.5% (w/v) deoxycholate, 1% (v/v) Nonidet P-40, and 2% (v/v) protease inhibitor cocktail (Sigma). Insoluble material was removed by centrifugation (13,000*g*, 10 min) and the intracellular uptake of [^{14}C]zoledronate in the soluble fraction was quantified using liquid scintillation counting. The insoluble pellet (which was not radioactive) was dissolved in 0.3 M NaOH. A protein assay (bicinchoninic acid assay; Sigma) was then carried out to allow determination of specific activity (expressed as picomoles of zoledronate per milligram of protein).

Western Blot Analysis. To determine whether the entry of bisphosphonate into the cytosol of the cells was affected by any of the compounds tested by flow cytometric analysis, J774 cells were treated with 100 μM alendronate or 100 μM zoledronate alone or in combination with 1 mM clodronate, 1 mM EGTA, 1 mM $CaCl_2$, 1 mM $SrCl_2$, or 1 mM $MgCl_2$ or with 250/500 μM dansylcadaverine, 10/20 μM monensin, or 50 nM bafilomycin A1 for 4 h. We also examined the effects of 1 mM $CaCl_2$, 1 mM $SrCl_2$, or 1 mM $MgCl_2$ on inhibition of Rap1A prenylation induced by treatment with 2.5 μM mevastatin or a geranylgeranyl transferase inhibitor (GGTI-298; 2.5 μM). The cells were lysed in 100 μl of radioimmunoassay precipitation buffer [1% Triton-X-100 (v/v), 0.5% sodium deoxycholate (w/v), 0.1% SDS (w/v) in PBS]. Because rabbit osteoclast-like cells generated in bone marrow cultures are less sensitive to the effects of alendronate than J774 cells (F. P. Coxon, J. C. Crockett, and M. J. Rogers, unpublished observations), we examined the effect of 250 μM dansylcadaverine, 10 μM monensin, and 25 nM bafilomycin A1 on alendronate-induced inhibition of Rap1A prenylation after 24 h of treatment (rather than after 4 h). The osteoclast-like cells were washed in PBS, then lysed in 100 μl of radioimmunoprecipitation assay buffer. After protein determination (bicinchoninic acid assay; Sigma), 30 μg of protein (J774 cells) or 50 μg of protein (osteoclast-like cells) from each lysate were electrophoresed under reducing conditions on a 12% polyacrylamide-SDS gel (Criterion electrophoresis system; Bio-Rad, Hemel Hempstead, UK). The proteins were transferred to polyvinylidene difluoride membrane, which was then incubated with 0.2 μg/ml goat polyclonal anti-Rap1A antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by 0.2 μg/ml anti-goat IgG-HRP conjugate (Merck BioSciences, Nottingham, UK). After visualizing the chemiluminescent signal, the membrane was then incubated in rabbit polyclonal anti-actin antibody (Sigma), followed by 0.2 μg/ml anti-rabbit IgG-HRP conjugate (Merck BioSciences). The chemiluminescence was visualized using a Bio-Rad FluorS MAX imager.

Statistical Analysis. Data were analyzed using a one-way analysis of variance with Tukey post hoc test.

Results

Ca^{2+} and Sr^{2+} Ions Stimulate AF-ALN Uptake by J774 Cells. To examine the pattern of uptake of AF-ALN, J774 cells that had been treated for 6 h with 100 μM AF-ALN were analyzed by confocal microscopy. Green fluorescence could be observed in punctate vesicles throughout the cytosol (Fig. 1A). The mean fluorescence per cell (the geomean) was quantitated by flow cytometry. When cells that had been treated for 1, 2, 3, and 4 h were analyzed, there was a time-dependent accumulation of fluorescence within the cells (Fig. 1B).

To determine the effect of divalent ions on AF-ALN uptake, J774 cells were treated with 100 μM AF-ALN in the presence or absence of 1 mM $CaCl_2$, 1 mM $SrCl_2$, or 1 mM $MgCl_2$. The mean amount of fluorescence per cell increased 23- and 10-fold when cells were treated for 4 h with 100 μM AF-ALN + 1 mM $CaCl_2$ or 100 μM AF-ALN + 1 mM $SrCl_2$, respectively, compared with treatment with AF-ALN alone. By contrast, when cells were coincubated with 1 mM $MgCl_2$ there was no additional increase in AF-ALN internalization (Fig. 1C). Similar results were obtained after quantifying the uptake of [^{14}C]zoledronate in the presence of $CaCl_2$, $SrCl_2$, or $MgCl_2$ (Fig. 1D). Furthermore, these observations were supported by Western blot analysis of J774 cells that had been treated for 4 h with 100 μM alendronate in the presence or absence of 1 mM $CaCl_2$, 1 mM $SrCl_2$, or 1 mM $MgCl_2$. As a result of inhibition of farnesyl diphosphate synthase, alendronate causes accumulation of unprenylated Rap1A in J774 cells, which can be detected by Western blot analysis using an antibody that specifically hybridizes to the unprenylated form of the small GTPase Rap1A (Coxon et al., 2001; Frith et al., 2001). After 4 h of treatment with alendronate, there was a slight accumulation of unprenylated Rap1A. However, this was markedly increased in cells treated with alendronate + $CaCl_2$ or alendronate + $SrCl_2$ but not in cells treated with alendronate + $MgCl_2$ (Fig. 1E). Unlike with alendronate, the addition of $CaCl_2$, $SrCl_2$, or $MgCl_2$ to the culture medium did not enhance the ability of either GGTI-298 or mevastatin to inhibit Rap1A prenylation (Fig. 1E).

EGTA and Clodronate Inhibit the Uptake and Action of Alendronate, Which Is Partially Reversed by Ca^{2+}. To investigate the requirement for Ca^{2+} ions in the uptake of AF-ALN, we examined the effect of the calcium chelator EGTA. J774 cells were treated for 4 h in the presence of 100 μM AF-ALN with or without 100 μM, 250 μM, 500 μM, or 1 mM EGTA. EGTA inhibited the uptake of AF-ALN into J774 cells in a significant and concentration-dependent manner and was effective at 100 μM, the lowest concentration tested, reducing the level of fluorescence per cell to 70% of the uptake of AF-ALN alone (data not shown). EGTA (1 mM) significantly reduced the fluorescence to 46% of the uptake of AF-ALN alone (Fig. 2A).

Because we have previously described an inhibitory effect of clodronate on the uptake of radiolabeled ibandronate (Frith and Rogers, 2003), and clodronate is an effective calcium chelator (by virtue of its two phosphonate groups), we also examined whether clodronate affected AF-ALN uptake by chelating Ca^{2+}. J774 cells were treated for 4 h in the

presence of 100 μM AF-ALN with or without 100 μM, 250 μM, 500 μM, or 1 mM clodronate. Like EGTA, clodronate inhibited the uptake of AF-ALN into J774 cells in a significant and concentration-dependent manner. Clodronate (100 μM) reduced the level of fluorescence per cell to 59% of the uptake of AF-ALN alone (data not shown). Clodronate (1 mM) reduced the fluorescence to 39% of the uptake of AF-ALN alone (Fig. 2A). The effects of both 1 mM EGTA and 1 mM clodronate on AF-ALN internalization were partially but significantly reversed (to 73% and 76%, respectively) by the addition of 1 mM $CaCl_2$. The same effect was also observed when we repeated these experiments using 25 μM radiolabeled zoledronate instead of AF-ALN and measured the amount of radioactivity internalized in the presence or absence of 1 mM EGTA or 1 mM clodronate (data not shown). To further clarify whether the inhibitory effect of clodronate on AF-ALN uptake was due to chelation of calcium ions, we repeated the above experiment using calcium-free DMEM. In

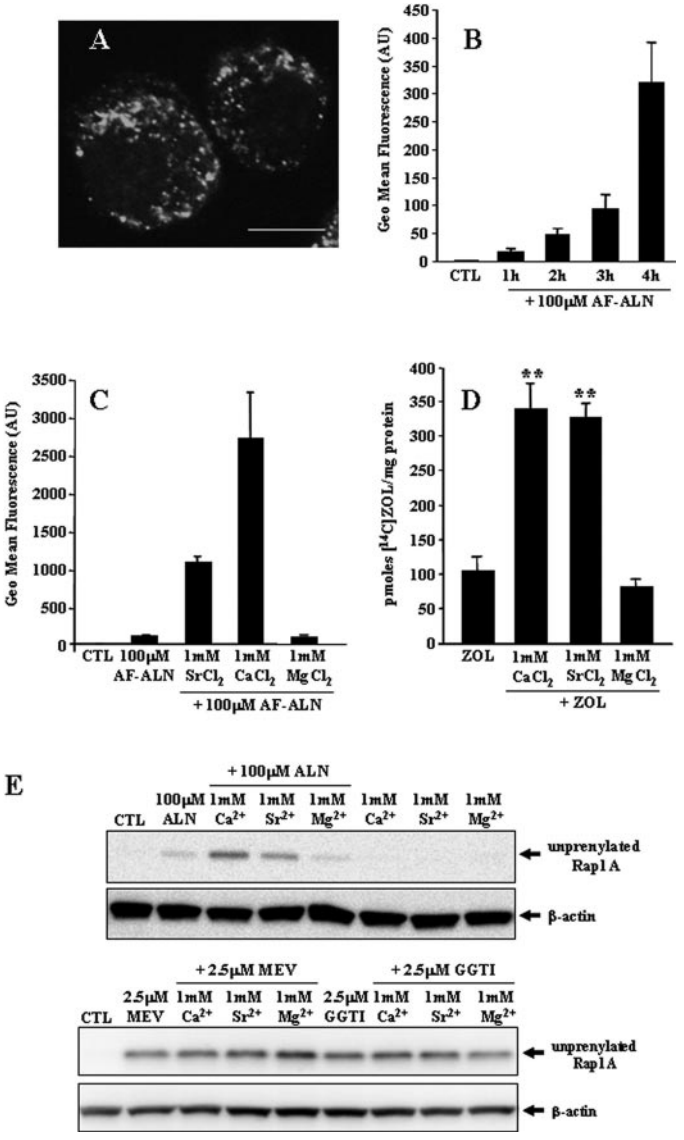

Fig. 1. Internalization of AF-ALN by J774 cells. A, J774 cells were incubated for 6 h in the presence of 100 μM AF-ALN, then fixed and analyzed by confocal microscopy (scale bar, 10 μm). Cells were incubated for 1 to 4 h with 100 μM AF-ALN (B) or for 4 h with 100 μM AF-ALN alone or with 1 mM $CaCl_2$, 1 mM $SrCl_2$, or 1 mM $MgCl_2$ (C), then fixed and analyzed by flow cytometry. Results shown are mean ($\pm$ S.E.M.) of four independent experiments. D, J774 cells were incubated for 4 h with 25 μM [^{14}C]zoledronate (ZOL) alone or with 1 mM $CaCl_2$, 1 mM $SrCl_2$, or 1 mM $MgCl_2$, lysed, then internalized [^{14}C]zoledronate was quantified by liquid scintillation counting. Results shown are mean ($\pm$ S.E.M.) of four independent experiments. **, $p < 0.01$ compared with 25 μM [^{14}C]zoledronate alone. E, cells were incubated for 4 h with 100 μM alendronate (ALN), 2.5 μM mevastatin (MEV), or 2.5 μM GGTI-298 $\pm$ 1 mM $CaCl_2$, 1 mM $SrCl_2$, or 1 mM $MgCl_2$ then analyzed by Western blotting for unprenylated Rap1A and β-actin. The data shown are representative of three independent experiments.

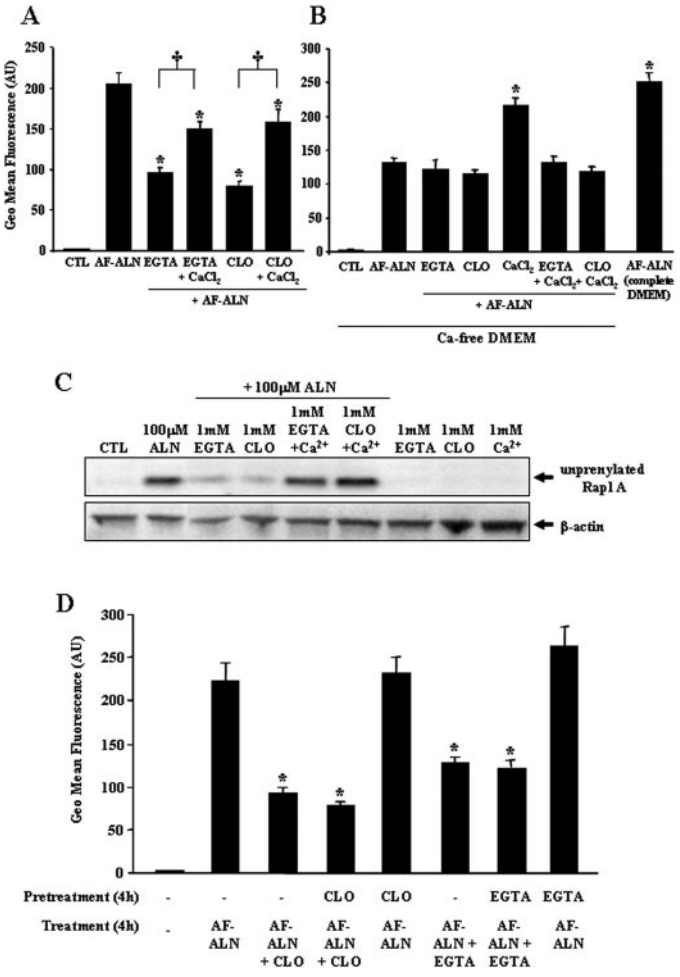

Fig. 2. Internalization of AF-ALN and alendronate into J774 cells is reduced by removal of Ca^{2+}. J774 cells were incubated for 4 h with 100 μM AF-ALN alone or in the presence of 1 mM EGTA or clodronate, $\pm$ 1 mM $CaCl_2$, in complete DMEM (containing 1.8 mM $CaCl_2$) (A) or calcium-free DMEM (B), then fixed and analyzed by flow cytometry. Results are mean ($\pm$ S.E.M.) of four independent experiments. *, $p < 0.05$ compared with 100 μM AF-ALN alone; †, $p < 0.05$ compared with treatments without $CaCl_2$. C, J774 cells were incubated with 100 μM alendronate (ALN), 1 mM clodronate (CLO), or 1 mM EGTA alone or with 100 μM alendronate $\pm$ 1 mM EGTA or 1 mM clodronate, in the presence or absence of 1 mM $CaCl_2$, then analyzed by Western blotting for unprenylated Rap1A and β-actin. The data shown are representative of three independent experiments. In the experiment shown, the ratio of unprenylated Rap1A to β-actin was 2.79 with 100 μM alendronate and was reduced to 1.00 and 0.71 by clodronate and EGTA, respectively. Ca^{2+} reversed this effect on Rap1A prenylation to ratios of 1.66 (EGTA) and 1.80 (clodronate). D, J774 cells were pretreated for 4 h with 1 mM EGTA, 1 mM clodronate, or PBS. The cells were then washed in fresh medium and incubated for 4 h with either 100 μM AF-ALN alone or with 1 mM EGTA or 1 mM clodronate, then fixed and analyzed by flow cytometry. Results are mean ($\pm$ S.E.M.) of three independent experiments. *, $p <$ 0.05 compared with treatment with AF-ALN for 4 h (with no pretreatment).

the latter culture medium, the uptake of AF-ALN by J774 cells was only 55% of that observed in complete DMEM (which contains 1.8 mM $CaCl_2$). Unlike in complete DMEM, 1 mM EGTA and 1 mM clodronate had little effect on the uptake of AF-ALN (Fig. 2B). Furthermore, addition of 1 mM $CaCl_2$ to calcium-free DMEM stimulated the uptake of AF-ALN to a level similar to that seen in complete DMEM, and this effect of Ca^{2+} was completely reversed in the presence of clodronate or EGTA (Fig. 2B).

In addition to measuring the inhibitory effect of EGTA and clodronate on AF-ALN uptake, we also examined their effect on alendronate-induced inhibition of Rap1A prenylation (a reflection of both the uptake of alendronate and the inhibition of farnesyl diphosphate synthase). EGTA (1 mM) or clodronate (1 mM) markedly reduced the inhibition of Rap1A prenylation caused by alendronate treatment (Fig. 2C). This effect was largely prevented when the cells were coincubated with 1 mM $CaCl_2$. EGTA, clodronate, or $CaCl_2$ alone had no effect on Rap1A prenylation (Fig. 2C).

To determine whether the inhibitory effects of EGTA and clodronate on alendronate uptake/accumulation of unprenylated Rap1A were indeed a result of Ca^{2+} chelation rather than an effect on cell metabolism or toxicity, J774 cells were pretreated with 1 mM clodronate or 1 mM EGTA for 4 h and then cultured for a further 4 h with AF-ALN, with or without clodronate or EGTA. Both EGTA and clodronate effectively inhibited uptake of AF-ALN only when present simultaneously with AF-ALN in the culture medium (Fig. 2D). When cells were only pretreated with EGTA or clodronate, the uptake of AF-ALN was not affected.

AF-ALN Colocalizes with Dextran but Not Wheat Germ Agglutinin or Transferrin. To determine whether AF-ALN is internalized by adsorptive endocytosis, receptor-mediated endocytosis or fluid-phase endocytosis, we examined whether AF-ALN colocalized with fluorescently labeled markers of each of these processes. J774 cells were incubated in the presence of 100 μM AF-ALN together with 10 μg/ml wheat germ agglutinin-633, 20 μg/ml transferrin-633, or 250 μg/ml TAMRA-dextran for 6 h. AF-ALN could be detected in intracellular vesicles but did not colocalize with wheat germ agglutinin-633 (Fig. 3Ai) or with transferrin-633 (Fig. 3Aii). However, AF-ALN at least partially colocalized in the same vesicles as TAMRA-dextran, a marker of fluid-phase endocytosis (Fig. 3Aiii).

Punctate, vesicular uptake of AF-ALN, similar to that seen in J774 cells, was also observed in mature rabbit osteoclasts (Fig. 3Aiv). This pattern of uptake closely resembled that seen with TAMRA-dextran (Fig. 3Av). Indeed, in rabbit osteoclasts, AF-ALN colocalized with TAMRA-dextran but not with transferrin-633 (Fig. 3Avi).

To further confirm that fluid-phase endocytosis, rather than adsorption or receptor binding, is involved in the uptake of AF-ALN, J774 cells were incubated for 1 h with 100 μM AF-ALN, 250 μg/ml FITC-dextran, 10 μg/ml wheat germ agglutinin-633, or 20 μg/ml transferrin-633 at 4°C or 37°C, then washed and incubated for 3 h at 37°C. The cells were then washed again, fixed, and analyzed by flow cytometry. The amount of fluorescence per cell was therefore a reflection of the amount of each compound bound to the cell surface during the 1-h incubation at 4°C or 37°C and later internalized at 37°C (Fig. 3B). Cells that had been treated with AF-ALN or FITC-dextran for 1 h at 4°C accumulated 3% and 0%, respectively, of the amount accumulated by cells that had been incubated for 1 h at 37°C. By contrast, cells treated with wheat germ agglutinin-633 or transferrin-633 at 4°C still accumulated 30% and 35%, respectively, of the amount internalized at 37°C, demonstrating that, unlike wheat germ agglutinin or transferrin, negligible amounts of AF-ALN or dextran bind to the cell surface.

Clodronate and EGTA Inhibit AF-ALN Uptake without Affecting Fluid-Phase Endocytosis. The effect of clodronate on the internalization of AF-ALN and TAMRA-dex-

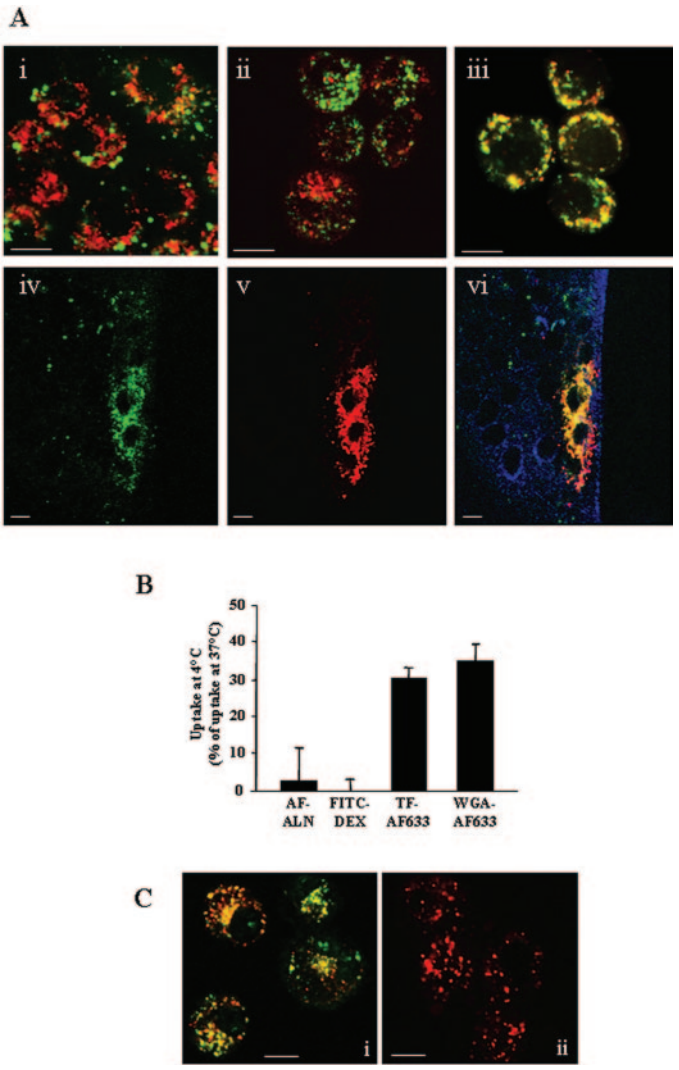

Fig. 3. AF-ALN is internalized by fluid-phase endocytosis in J774 cells and in rabbit osteoclasts. A, J774 cells (i–iii) and rabbit osteoclasts (iv–vi) were incubated with 100 μM AF-ALN + 1 μg/ml wheat germ agglutinin-633 (i), 100 μM AF-ALN + 20 μg/ml transferrin-633 (ii), 100 μM AF-ALN + 250 μg/ml TAMRA-dextran (iii), 100 μM AF-ALN (iv), 250 μg/ml TAMRA-dextran (v), or 100 μM AF-ALN + TAMRA-dextran + transferrin-633 (vi) for 6 h, then fixed and analyzed by confocal microscopy (scale bar, 10 μm). B, J774 cells were incubated with 100 μM AF-ALN, 250 μg/ml FITC-dextran (FITC-DEX), 10 μg/ml wheat germ agglutinin-633 (WGA-AF633), or 20 μg/ml transferrin-633 (TF-AF633) at 4°C or 37°C for 1 h. The cells were then washed, incubated for a further 3 h in treatment-free medium, then fixed and analyzed by flow cytometry. Results are presented as uptake after incubation at 4°C as a percentage of the uptake after incubation at 37°C and are mean (± S.E.M.) of four independent experiments. C, J774 cells were incubated for 6 h with 250 μg/ml TAMRA-dextran + 100 μM AF-ALN, in the presence or absence of 1 mM clodronate, then fixed and analyzed by confocal microscopy (scale bar, 10 μm).

tran by J774 cells was visualized by confocal microscopy. Cells were treated with 250 μg/ml TAMRA-dextran together with 100 μM AF-ALN in the presence or absence of 1 mM clodronate. TAMRA-dextran and AF-ALN colocalized within the same vesicles (Fig. 3Ci). When the cells were coincubated with 1 mM clodronate, internalization of AF-ALN was prevented, but the uptake of TAMRA-dextran was unaffected (Fig. 3Cii). Furthermore, when the uptake of FITC-dextran over 4 h was quantified by flow cytometry, 1 mM clodronate or 1 mM EGTA (which significantly inhibited the uptake of AF-ALN; Fig. 2A) had no effect on the uptake of FITC-dextran, whereas 1 mM $CaCl_2$ (which significantly stimulated the uptake of AF-ALN or [^{14}C]zoledronate; Fig. 1, C and D) had no effect on the uptake of FITC-dextran (data not shown). Together, these observations demonstrate that chelation of Ca^{2+} ions by clodronate or EGTA inhibits the uptake of bisphosphonates by a physicochemical mechanism rather than by reducing fluid-phase endocytosis.

Dansylcadaverine Inhibits Endocytic Uptake of AF-ALN and [^{14}C]Zoledronate. To examine the effect of dansylcadaverine, monensin, and bafilomycin A1 on fluid-phase endocytosis, J774 cells were treated for 4 h with 250 μg/ml FITC-dextran in the presence or absence of 500 μM dansylcadaverine, 20 μM monensin, or 50 nM bafilomycin A1. Consistent with its known inhibitory effect on endocytosis (Haigler et al., 1980), dansylcadaverine significantly reduced the uptake of dextran by almost 75% (Fig. 4A). However, neither monensin nor bafilomycin A1 had any significant effect. Likewise, 500 μM dansylcadaverine significantly reduced the uptake of AF-ALN to 50% of that observed with AF-ALN alone (Fig. 4B), whereas neither monensin nor bafilomycin A1 inhibited the uptake of AF-ALN. The same effects were observed on the uptake of [^{14}C]zoledronate; 500 μM dansylcadaverine significantly reduced the uptake of [^{14}C]zoledronate, whereas monensin or bafilomycin A1 had no effect (Fig. 4C).

To confirm the lack of effect of bafilomycin A1 and monensin on vesicular uptake of AF-ALN, J774 cells that had been treated with AF-ALN with or without monensin or bafilomycin A1 were also examined by confocal microscopy. Consistent with the quantification of uptake of FITC-dextran, AF-ALN or [^{14}C]zoledronate (Fig. 4, A–C), monensin or bafilomycin A1 did not affect endocytosis of AF-ALN into punctate vesicles (Fig. 4D).

Dansylcadaverine, Monensin, and Bafilomycin A1 Prevent Bisphosphonate-Induced Inhibition of Rap1A Prenylation. By contrast with the measurement of AF-ALN uptake by flow cytometry, which gives an indication of the total amount of intracellular AF-ALN (e.g., cytosolic *and* vesicular), analysis of Rap1A prenylation demonstrates effects on exit of bisphosphonates from the endosomes into other intracellular compartments where farnesyl diphosphate synthase resides. Farnesyl diphosphate synthase is synthesized within the cytosol and is translocated post-translationally into peroxisomes (Olivier et al., 2000). In agreement with the flow cytometric analyses, dansylcadaverine partially prevented the alendronate-induced accumulation of unprenylated Rap1A in a concentration-dependent manner (Fig. 5A). The same effect was observed when J774 cells were treated with 100 μM zoledronate + dansylcadaverine. Like alendronate, 100 μM zoledronate caused an accumulation of unprenylated Rap1A, which was partially, and concentration dependently, prevented when the cells were coincubated with 250 or 500 μM dansylcadaverine (Fig. 5A).

In contrast to the lack of effects seen on the uptake of AF-ALN, either monensin or bafilomycin A1 completely prevented the accumulation of unprenylated Rap1A when J774 cells were treated with 100 μM alendronate or zoledronate (Fig. 5A). J774 cells accumulated the acidotropic probe LysoTracker Red (Molecular Probes) within intracellular vesicles in untreated J774 cells, but not in cells treated with 50 nM bafilomycin A1 or 20 μM monensin (data not shown), indicating that both bafilomycin A1 and monensin, at concentrations that blocked the inhibitory effect of N-BPs on protein prenylation, prevented the acidification of intracellular organelles.

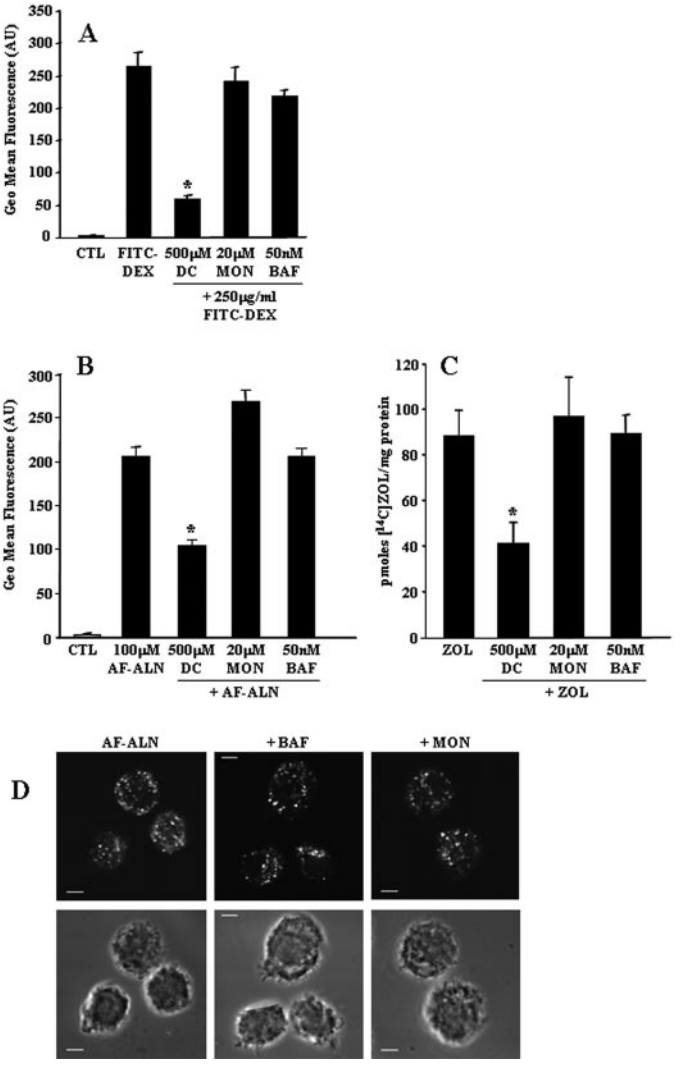

Fig. 4. Internalization of alendronate is modified by dansylcadaverine, monensin and bafilomycin A1. J774 cells were incubated for 4 h with 250 μM FITC-dextran (A), 100 μM AF-ALN (B), or 25 μM [^{14}C]zoledronate (C), ± 500 μM dansylcadaverine (DC), 20 μM monensin (MON), or 50 nM bafilomycin A1 (BAF). Cells were then fixed and analyzed by flow cytometry (A and B) or lysed, then internalized [^{14}C]zoledronate was quantified by liquid scintillation counting (C). Results are mean (± S.E.M.) of four independent experiments. $*= p < 0.05$ compared with treatment with FITC-dextran, AF-ALN, or [^{14}C]zoledronate alone. D, J774 cells were incubated for 6 h with 100 μM AF-ALN ± 20 μM monensin or 50 nM bafilomycin A1, then fixed and analyzed by confocal microscopy (scale bar, 10 μm).

In agreement with the data obtained in J774 cells, 10 μM monensin, 25 nM bafilomycin A1 or 250 μM dansylcadaverine prevented the inhibitory effect of alendronate on Rap1A prenylation in rabbit osteoclast-like cells (Fig. 5B).

Discussion

Bisphosphonate drugs are effective calcium chelators and, after oral or i.v. administration, rapidly target the skeleton, where they inhibit osteoclast-mediated bone resorption (Rogers, 2003). During bone resorption, acid (H^+ and Cl^- ions) and proteolytic enzymes are actively secreted across the osteoclast ruffled border membrane (Sundquist et al., 1990). In the acidic pH of the resorption lacuna, protonation of the phosphonate groups of bisphosphonates is believed to cause their release from bone surfaces into solution (Ebetino et al., 1998). However, the exact route by which bisphosphonate drugs are then internalized by osteoclasts (and possibly other cells in the bone microenvironment) remains unknown. In this study, we examined the mechanism of internalization of bisphosphonates by studying the uptake of radiolabeled zoledronate and a novel, fluorescently labeled analog of alendronate (AF-ALN) into J774 macrophages and rabbit osteoclasts. J774 cells were used because the structure-activity relationships of bisphosphonates for reducing J774 cell viability closely matches the structure-activity relationships for inhibiting bone resorption in vivo (Luckman et al., 1998a) as a result of effects on the same intracellular molecular target, farnesyl diphosphate synthase (Dunford et al., 2001).

In macrophages and osteoclasts, AF-ALN seemed to be internalized from solution initially by fluid-phase endocytosis, because the punctate, vesicular fluorescence staining (Fig. 1) colocalized with a marker of fluid-phase endocytosis (FITC-dextran) but not with markers of adsorptive or receptor-mediated endocytosis (wheat germ agglutinin or transferrin, respectively) (Fig. 3). As with FITC-dextran, we could not detect appreciable binding of AF-ALN to cells incubated at 4°C (unlike wheat germ agglutinin or transferrin), suggesting the lack of any receptor-binding or adsorptive step before endocytosis of AF-ALN (Fig. 3). The rapid internalization of AF-ALN, and continued accumulation over several hours (Fig. 1), is also consistent with the rapid rate of fluid-phase endocytosis of these cells. Furthermore, the vesicular uptake of AF-ALN was at least partially prevented by an inhibitor of fluid-phase endocytosis, dansylcadaverine (Davies et al., 1980; Schlegel et al., 1982), which also prevented uptake of FITC-dextran (Fig. 4). This route of uptake of AF-ALN was not an artifact because of modification of a bisphosphonate by addition of a bulky fluorophore; dansylcadaverine also prevented the internalization of [^{14}C]zoledronate and concentration-dependently prevented the inhibitory effect of two unlabeled N-BPs (alendronate and zoledronate) on the prenylation of Rap1A. The latter effect involves inhibition of the target enzyme farnesyl diphosphate synthase, which is synthesized in the cytosol and translocated, probably as the folded protein, into peroxisomes (McNew and Goodman, 1994; Olivier et al., 2000). Together, these observations demonstrate that initial internalization by fluid-phase endocytosis is a prerequisite for the pharmacological action of N-BPs.

To determine how N-BPs translocate from endocytic vesicles into the cytoplasm, we examined the effect of inhibitors of endosomal acidification. Monensin is an ionophore that prevents acidification of endosomes (Kaiser et al., 1988) and bafilomycin A1 is an inhibitor of the vacuolar H^+-ATPase (Bowman et al., 1988). Neither monensin nor bafilomycin A1 had any detectable inhibitory effect on the total uptake of AF-ALN, FITC-dextran, or [^{14}C]zoledronate into J774 cells (Fig. 4). However, monensin and bafilomycin A1 both prevented the inhibitory effect of alendronate or zoledronate on Rap1A prenylation in macrophages and osteoclasts. This demonstrates that although monensin and bafilomycin A1 do not inhibit the internalization of bisphosphonate into intracellular vesicles, they prevent entry of the drugs into the cytosol by preventing acidification of endocytic vesicles. At low pH, protonation of the phosphonate groups of bisphosphonates would reduce the anionic charge of the compounds, thereby releasing any bound Ca^{2+} ions and probably allowing diffusion of the bisphosphonates across the vesicular membrane into the cytoplasm (although we cannot rule out the possibility that bisphosphonates traverse the vesicular membrane via a transport protein). Our observation that bafilomycin A1 and monensin prevent the inhibitory effect of alendronate or zoledronate on protein prenylation in macrophages and osteoclasts in vitro are consistent with a recent study demonstrating that bafilomycin A1 prevented the disruptive effect of the bisphosphonates risedronate and etidronate on cytoskeletal organization of osteoclasts in vitro (Takami et al., 2003). However, in the latter study, the authors concluded incorrectly that extracellular acidification was necessary for internalization of bisphosphonates across the plasma membrane of osteoclasts in the absence of bone

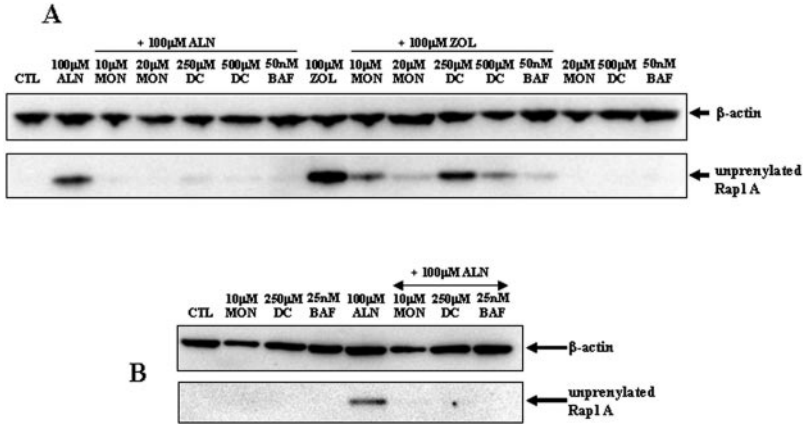

Fig. 5. Dansylcadaverine, monensin, and bafilomycin A1 prevent the inhibitory effect of alendronate or zoledronate on protein prenylation. J774 cells (A) or rabbit osteoclast-like cells (B) were treated for 4 h (A) or 24 h (B) with 100 μM alendronate (ALN) or zoledronate (ZOL), ± 250/500 μM dansylcadaverine (DC), 10/20 μM monensin (MON) or 25/50 nM bafilomycin A1 (BAF). Cell lysates were analyzed by western blotting for unprenylated Rap1A and β-actin. Data are representative of three independent experiments.

mineral. We clearly demonstrate that, in fact, internalization by fluid-phase endocytosis (which is inhibited by dansylcadaverine) is required first, followed by acidification of endocytic vesicles.

The lack of an effect of bafilomycin A1 or monensin on the total amount of intracellular AF-ALN or [^{14}C]zoledronate (Fig. 4), despite complete inhibition of the effects of alendronate or zoledronate on protein prenylation (Fig. 5), indicates that the amount of N-BP in the cytosol is extremely low compared with the amount present in endocytic vesicles. Because N-BPs such as zoledronate inhibit farnesyl diphosphate synthase at nanomolar and even picomolar concentrations (Dunford et al., 2001), very low concentrations achieved in the cytosol must still be sufficient to inhibit farnesyl diphosphate synthase in peroxisomes. This enzyme is synthesized in the cytosol on free ribosomes and then imported into peroxisomes. Most proteins destined for the peroxisomal matrix are translocated in their mature conformations (McNew and Goodman, 1996). It is possible, therefore, that bisphosphonates associate with farnesyl diphosphate synthase in the cytosol and are then imported into the peroxisomes together with newly synthesized farnesyl diphosphate synthase. Some proteins that lack a specific peroxisome targeting sequence have also been demonstrated to "piggy-back" onto other proteins to gain access to the peroxisomes (McNew and Goodman, 1994).

Finally, because a previous study by Mönkkönen et al. (1994) demonstrated that Ca^{2+} ions enhanced the inhibitory effect of bisphosphonates on RAW264 cell proliferation, we examined the role of Ca^{2+} ions on the uptake of N-BPs. Consistent with these previous observations, we found that the presence of 1 mM Ca^{2+} or Sr^{2+} (but not Mg^{2+}) increased the uptake of AF-ALN or [^{14}C]zoledronate and enhanced the inhibitory effect of alendronate on protein prenylation (Fig. 1). Furthermore, addition of EGTA or a molar excess of the bisphosphonate clodronate, which does not inhibit protein prenylation (Luckman et al., 1998b; Dunford et al., 2001), significantly reduced (but did not entirely inhibit) the uptake of AF-ALN and reduced the inhibitory effect of alendronate on protein prenylation (Fig. 2). The latter effects of EGTA or clodronate were due to chelation of Ca^{2+}, because clodronate or EGTA had to be present simultaneously with the AF-ALN and the effects could be overcome by the further addition of Ca^{2+}(Fig. 2). In addition, when J774 cells were cultured in calcium-free medium, AF-ALN uptake decreased to approximately half of that seen in complete DMEM and was not decreased further by the addition of clodronate or EGTA. Furthermore, clodronate did not affect the uptake of FITC-dextran (Fig. 3). Together, these observations demonstrate that the presence of Ca^{2+} or Sr^{2+} ions enhances the endocytic internalization of N-BPs by some physicochemical mechanism. Indeed, it has been reported that Ca^{2+} promotes the aggregation of precipitable, polymeric complexes with bisphosphonates (Matczak-Jon et al., 2002). This also explains our earlier finding that clodronate could prevent the inhibitory effect of the N-BP ibandronate on protein prenylation (Frith and Rogers, 2003) by chelating Ca^{2+} and reducing ibandronate uptake. Unlike Ca^{2+} and Sr^{2+} ions, Mg^{2+} did not seem to stimulate uptake of AF-ALN (Fig. 1) or enhance the effect of alendronate on Rap1A prenylation. Although bisphosphonates can chelate Mg^{2+} ions, the latter are less likely to form multinuclear complexes with bisphosphonates because Mg^{2+} ions are smaller and less flexible than Ca^{2+} (Matczak-Jon and Videnova-Adrabinska, 2005).

In conclusion, we provide the first conclusive evidence that cellular internalization of bisphosphonate drugs is dependent on fluid-phase endocytosis and vesicular acidification. In vivo, bisphosphonates are rapidly cleared from the circulation (Lin, 1996) and bind to Ca^{2+}-containing bone mineral surfaces at sites of active bone remodelling, particularly areas undergoing osteoclastic resorption (Masarachia et al., 1996). Because the ability to chelate Ca^{2+} is reduced at acidic pH (Ebetino et al., 1998), bisphosphonate bound to bone mineral is released from the bone surface in the acidic environment of the resorption lacuna beneath the resorbing osteoclast, giving rise to a concentrated, localized drug solution (Rogers, 2003). Our findings suggest that bisphosphonate is probably then internalized into osteoclasts (perhaps as complexes with Ca^{2+}) by fluid-phase endocytosis of the extracellular fluid. Consistent with this, radiolabeled bisphosphonate has been detected by microautoradiography within endocytic vacuoles in resorbing osteoclasts (Sato et al., 1991; Masarachia et al., 1996). Vacuolar-type H^+-ATPase is also highly abundant in osteoclasts (Vaananen et al., 1990), thereby allowing acidification of endocytic vesicles and entry of bisphosphonate into the osteoclast cytosol.

In addition to their known ability to affect osteoclasts, there is considerable interest in the potential antitumor activity of N-BPs in vivo, via direct effects on tumor cells or on other tumor-associated cells such as endothelial cells or infiltrating macrophages (Giraudo et al., 2004; Green, 2004). Although bisphosphonates can affect a wide variety of cell types in vitro (Rogers, 2003; Green, 2004), our studies suggest that the ability of bisphosphonates to affect cells other than osteoclasts in vivo may be determined by their endocytic capacity, as well as by the concentration of available bisphosphonate in the extracellular fluid.

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