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Clodronate-Induced Cell Apoptosis in Human Thyroid Carcinoma Is Mediated via the P2 Receptor Signaling Pathway

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ABSTRACT

Clodronate, a halogenated bisphosphonate, can inhibit the growth of human thyroid carcinoma (TC) cells. Previously, we found that a clodronate-induced Ca^{2+} transient was correlated with clodronate-induced growth inhibition in TC cells. However, the details of the signaling process underlying the antiproliferative effect of clodronate on TC cells are not clear. In this study, we investigated the antiproliferative mechanism of clodronate on papillary TC (PTC) cells and xenotransplanted animals using a combination of pharmacological drugs. Reverse transcription-polymerase chain reaction analysis confirmed the endogenous expression of P2Y receptor isoforms in PTC cells. The P2 antagonist suramin not only inhibited the antiproliferative effect of clodronate and ATP on TC cells but also blocked all the Ca^{2+} transients induced by clodronate and ATP. The release of Ca^{2+} from the endoplasmic reticulum and membrane depolarization of mitochondria was observed during the clodronate-induced Ca^{2+} transients. The results of terminal deoxynucleotidyltransferase dUTP nick-end labeling assays and flow cytometry with annexin V and caspase-3 staining suggest that both ATP and clodronate induce apoptosis. Significant inhibition of tumor invasion and colony formation was also observed in clodronate-treated PTC cells. We further demonstrated that only the cAMP inhibitor 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine (SQ22536), and not inhibitors of phospholipase C [1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122)] or store-operated Ca^{2+} entry (2-aminoethyl diphenylborinate), can significantly reverse the effect of clodronate. Finally, in vivo animal and green fluorescent protein imaging studies further proved that the tumor inhibitory effect of clodronate on xenotransplanted CG3 cells can be reversed by treatment with suramin. In conclusion, we demonstrated that clodronate-induced PTC cell apoptosis and tumor inhibition are partially mediated by the P2Y receptor-cAMP cascade.

Papillary thyroid carcinoma (PTC) is one of the most commonly differentiated thyroid carcinomas (TCs) (Kondo et al., 2006; Kung et al., 2006). Genomic research has revealed that RET rearrangement and BRAF mutation are important in the carcinogenesis of TC/PTC (Lee et al., 1998; Kondo et al., 2006). Thus far, the treatment choice for TC is surgery in combination with postoperative radioiodine therapy (Huang et al., 2005). However, in patients with postoperative recurrent or lymphoid metastatic lesions without radioiodative uptake, the success of TC therapy is limited (Huang et al., 2005; Muresan et al., 2008).

Anticancer or chemotherapeutic drugs are limited in effect and duration of response for the treatment of TC (Muresan et al., 2008). Recently, bisphosphonates (BPs) were found to have a broad range of in vivo and in vitro antitumor effects

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ABBREVIATIONS: PTC, papillary thyroid carcinoma; TC, thyroid carcinoma; BP, bisphosphonate; TMRE, tetramethylrhodamine ethyl ester; ER, endoplasmic reticulum; RP-mito, mitochondrial-targeted ratiometric pericam; MTT, dimethylthiazol-diphenyltetrazolium bromide; TG, thapsigargin; U73122, 1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; SQ22536, 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine; RT-PCR, reverse transcription-polymerase chain reaction; MT, mitochondria; Ψ_{MT}, mitochondrial membrane potential; TUNEL, terminal deoxynucleotidyltransferase dUTP nick-end labeling; GFP, green fluorescent protein; PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; PKA, protein kinase A; SOCE, store-operated Ca^{2+} entry; 2-APB, 2-aminoethyl diphenylborinate; N-BP, nitrogen-containing bisphosphonate; Smac/DIABLO, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low p*I*.

(Brown et al., 2004; Yang et al., 2004). Oral clodronate, one of the BPs, was shown to improve survival of breast cancer patients and to prevent bone metastasis (Diel et al., 1998, 2008). It was reported that an ATP analog formed from metabolized clodronate entering the cytosol with the help of liposome could be the cause of cell apoptosis (Lehenkari et al., 2002). We found previously that clodronate induces cell growth arrest and concomitantly generates a unique Ca^{2+} response in differentiated TC cell lines (Yang et al., 2004). However, it remains unclear whether the antitumor effect of clodronate on TC is mediated through this metabolized ATP analog mechanism (Lehenkari et al., 2002), the Ca^{2+} signaling cascade as reported in our previous study (Yang et al., 2004), or alternative pathways such as the purinergic receptor signaling pathway.

Extracellular ATP and related nucleotides are not only energy components but also important signaling molecules in purinergic receptor signaling (White and Burnstock, 2006). Most normal human cells express various isoforms of purinergic receptors for specific physiological functions (Dixon et al., 2004; Balogh et al., 2005; Inoue et al., 2007). Yet, it has also been reported that ATP induces apoptosis or growth inhibition of different cancer cells, including human melanoma (White et al., 2005), intestinal epithelial carcinoma (Coutinho-Silva et al., 2005), prostate cancer (Shabbir et al., 2008a), and bladder cancer (White and Burnstock, 2006; Shabbir et al., 2008b). To date, it is not known whether ATP or related drugs can induce TC cell death. The purpose of this study is to investigate the ATP- and/or clodronate-induced anticancer effect and its related mechanism.

Materials and Methods

Reagents, Cell Culture, and Transfection. Reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless indicated otherwise. Clodronate was purchased from Leiras Oy (Turku, Finland). The acetoxymethyl ester form of fura-2, ER-Tracker Green, and tetramethylrhodamine ethyl ester (TMRE) were from Invitrogen (Carlsbad, CA). The pDsRed2-mito-plasmid was obtained from Clontech (Mountain View, CA).

The PTC cell line CG3 was kindly provided by Dr. Jen-Der Lin (Chang Gung Memorial Hospital, Taipei, Taiwan, Republic of China) and was cultured as described previously (Lin et al., 1996). The use of primary culture human PTC cells was approved by the Institutional Review Board at Taipei Veterans General Hospital. In total, 28 PTC primary cell cultures were established from patient thyroid tumor samples after surgical ablation of PTC and cultured as described previously (Lin et al., 1996).

CG3 thyroid cancer cells were transfected with plasmids containing the ER-targeted D1ER or mitochondrial-targeted ratiometric pericam (RP-mito) using the Lipofectamine 2000 reagent (Invitrogen), as described previously (Chang et al., 2008). The transfected cells were used for the experiments after 2 days.

Cell Viability Assay. The measurement of cell viability was performed using the dimethylthiazol-diphenyltetrazolium bromide (MTT) method as described previously (Peng et al., 2008). CG3 cells were seeded on 24-well plates at a density of 2×10^4 cells/well in medium, followed by treatment with 0.25 mg/ml MTT for 4 h at 37°C. The reaction was terminated by the addition of 100% isopropanol. The amount of MTT was determined using a microplate reader, and the absorbance was measured at 560 nm (SpectraMax 250; Molecular Devices, Sunnyvale, CA). For treatment with various concentrations of drugs such as clodronate, ATP, or cisplatin with or without pharmacological inhibitors, the cells were bathed in cell culture medium containing the agents for the indicated time period. The pharmacological inhibitors used included suramin (200 μM), thapsigargin (TG; 1 μM), U73122 (0.5 μM), and SQ22563 (100 μM).

Reverse Transcription-PCR. RT-PCR of the P2Y receptor isoforms from CG3 cells was performed according to previously described procedures that included the primer design and the general protocol for RNA extraction and cDNA synthesis (Elia et al., 2003; Chang et al., 2008). Finnzymes PCR reagents (Finnzymes Oy, Espoo, Finland) were used in the RT-PCR reactions.

Measurements of $[Ca^{2+}]_i$, $[Ca^{2+}]_{ER}$, $[Ca^{2+}]_{MT}$, Ψ_{MT}, and Organelle Imaging. For cytosolic, ER, and mitochondrial Ca^{2+} as well as mitochondrial membrane potential (Ψ_{MT}) measurements, fluorescence measurements were conducted on fura-2 acetoxymethyl ester- (5 μM; Yang et al., 2004), D1ER- (Palmer et al., 2004), RP-mito- (Nagai et al., 2001), and TMRE (10 nM)-stained/transfected PTC cells using a time-lapse microscopy system (IX-71; Olympus, Tokyo, Japan) equipped with a 40× oil objective (numerical aperture, 1.35; U/340) and a monochromator (Polychrome II; TILL Photonics GmbH, Gräfelfing, Germany) driven by SimplePCI 6.0 software (Compix Institute, Sewickly, PA). A high-speed cooled charge-coupled device camera (MicroMAX: 782YHS; Roper Scientific, Trenton, NJ) was used to illuminate/acquire emission imaging of these fluorescent probes (340 and 380/505–520 nm for fura, 440/535 long pass for D1ER, 440/500–525 nm for RP-mito, and 540/560 long pass for TMRE). The intensity values of D1ER, TMRE, or RP-mito (F or F_{440}) were normalized by selecting the data over the first 10 s before drug application as the basal level (F_0). F/F_0 and $1 - F_{440}/F_0$ (the upward of reversed plot indicates the increase of mitochondrial Ca^{2+}) values were calculated.

The morphology and subcellular localization of mitochondria and ER of PTC cells were observed in cells stained with pDsRed2-mito/TMRE (mitochondria) and D1ER/ER-Tracker Green (ER) under a confocal laser scanning microscopy (LSM 5 Pa; Carl Zeiss, Jena, Germany).

Apoptosis Assays. Flow cytometry was used for cell apoptosis identification. A single cell suspension of cells (passages 6–8) was stained with fluorescein (fluorescein isothiocyanate)-tagged caspase-3 or fluorescein isothiocyanate-annexin V with propidium iodine. Cells were fixed with 2% paraformaldehyde and analyzed with an FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ).

Apoptotic cells were further confirmed via the terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL) method (In Situ Cell Death Detection Kit, POD; Roche Diagnostics, Indianapolis, IN) as described previously (Peng et al., 2008). In brief, the cells on coverslips were washed with 1× phosphate-buffered saline, fixed with 4% of paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 5 min, and incubated with the TUNEL reagent for 1 h. Chromogenic development was then applied with 3-amino-9-ethyl-carbazole. The percentage of TUNEL-positive cells was measured by the average cell number from six different areas in comparison with the total cell number in the same section of the slide (under a light microscope with a 20× objective).

In Vitro Cell Invasion Analysis and Soft Agar Assay. The 24-well plate Transwell system with a polycarbonate filter membrane (8-μm pore size; Dow Corning Ltd., Barry, Vale of Glamorgan, UK) was used. Cell suspensions were seeded in the upper compartment of the Transwell chamber at a density of 1×10^5 cells in 100 μl of serum-free medium. The opposite surface of the filter membrane, which faces the lower chamber, was stained with Hoechst 33342 dye for 3 min, and migrating cells were visualized under an inverted microscope. The soft agar assay was performed as follows: the bottom of each well (35 mm) of a six-well culture dish was coated with 2 ml of agar-medium mixture [Dulbecco's modified Eagle's medium, 10% (v/v) fetal calf serum, and 0.6% (w/v) agar]. After the bottom layer solidified, 2 ml of top agar-medium mixture [Dulbecco's modified Eagle's medium, 10% (v/v) fetal calf serum, and 0.3% (w/v) agar] containing 2×10^4 cells was added, and the samples were incubated at 37°C for 4 weeks. The plates were stained with 0.5 ml of 0.005%

crystal violet, and the number of colonies was counted using a dissecting microscope (Chen et al., 2008).

In Vivo Analysis of Tumor Growth and Metastasis. All procedures involving animals were in accordance with the institutional animal welfare guidelines of Taipei Veterans General Hospital and United Kingdom Coordinating Committee on Cancer Research (Workman et al., 1998). Cells (2×10^6) were injected into the subcutaneous neck site of 8-week-old male nude mice (BALB/c strain). After 1 week of CG3 cell xenotransplantation, the dosage of clodronate (20 mg/kg i.p., twice a week) was given to tumor-bearing mice (for each group, $n = 8$). To further block the treatment effect of clodronate, the administration of suramin (10 mg/kg i.p., twice a week) was combined with 20 mg/kg clodronate and given to each mouse. In vivo green fluorescent protein (GFP) imaging was visualized and measured by an illuminating device [LT-9500 Illumatool TLS equipped with an excitation illuminating source (470 nm) and a filter plate (515 nm)]. The tumor size was measured with calipers, and the tumor volume was calculated according to the formula (length $\times$ width^2)/2 (Geran et al., 1972). In addition, to further monitor the toxicity of clodronate alone ($n = 8$) and in combination with suramin ($n = 8$) in treated mice, the body weights of the mice were measured every week during the treatment. All of tumor-bearing nude mice were sacrificed after the 4-week study. The integrated optical density of green fluorescence intensity was captured and then analyzed by Image-Pro Plus software (MediaCybernetics, Inc., Bethesda, MD) as described previously (Yang et al., 2008).

Statistical Analysis. Data are expressed as means ± S.E.M. All data in this study were obtained from at least three independent experiments. For cytosolic Ca^{2+} increases, the peak values of the fura-2 ratio were calculated and analyzed by normalizing the image data against the basal level (the first 10 s before drug application) as the F_{340}/F_{380} ratio (fura-2). Changes were considered significant when a p value <0.05 was obtained using the Student's t test. The p value notation was set as $*$ for $p < 0.01$, $**$ for $p < 0.001$, and $***$ for $p < 0.0001$.

Results

Differential Inhibitory Effect of ATP, Clodronate, and Cisplatin on Proliferation of CG3 Papillary Thyroid Cancer Cells. The viability of CG3 cells was significantly decreased (50%) by ATP at a concentration of 1 mM after a 48-h treatment (Fig. 1A). Compared with ATP, clodronate exerted significant growth inhibition at 0.5 mM after the 48-h treatment (50%; Fig. 2B). The chemotherapeutic cisplatin significantly decreased CG3 cell proliferation at a concentration of 1 μM after the 48-h treatment (50%; Fig. 1C).

We tested the effect of the P2 receptor antagonist suramin (200 μM) on inhibition of drug-induced proliferation of CG3 cells after the 48-h treatment (Fig. 1, ○). The antiproliferation effect of 1 mM ATP was significantly attenuated by 200 μM suramin (Fig. 1A). The clodronate-induced anticancer growth effect was observed at a concentration of 0.25 mM (Fig. 1B). The anticancer effect of cisplatin was further enhanced by suramin (Fig. 1C). These results suggest that ATP, clodronate, and cisplatin decreased the proliferation of CG3 cells in a dose- and time (data not shown)-dependent manner. Furthermore, the P2 purinergic receptor signaling pathway was involved in the anti-CG3 proliferation effect of ATP and clodronate but not of cisplatin.

The Purinergic P2Y-PLC Pathway Exists in PTC Cells, and the ER Ca^{2+} Release Is Triggered by ATP or Clodronate. The above-mentioned data (Fig. 1) imply that clodronate and ATP may turn on the same signaling process

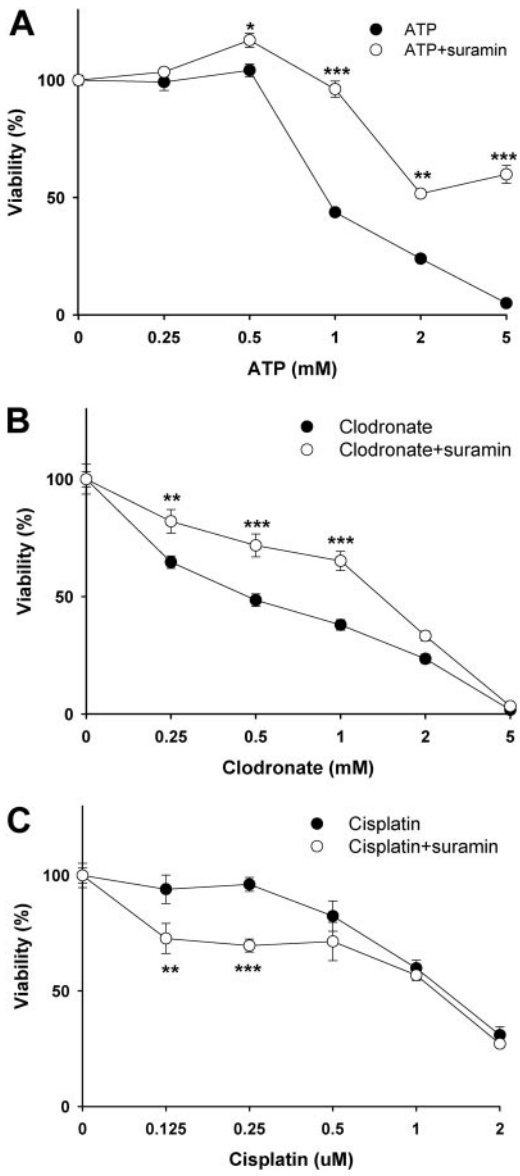

Fig. 1. ATP, clodronate, and cisplatin have an antiproliferative effect on CG3 thyroid cancer cells. The viability of CG3 cells under different concentrations of ATP (A; 0.25, 0.5, 1, 2, and 5 mM), clodronate (B; 0.25, 0.5, 1, 2, and 5 mM), or cisplatin (C; 0.125, 0.25, 0.5, 1, and 2 μM) was evaluated after a 48-h treatment (○). The ATP-induced cell growth inhibition was significantly detected at a concentration of 1 mM and reversed by 200 μM P2Y receptor antagonist suramin (●). Clodronate-induced cell growth inhibition was significantly detected at a concentration of 0.5 mM and reversed by suramin (●). Cisplatin-induced cell growth inhibition was significantly detected at a concentration of 1 μM, and suramin further enhanced this inhibition at cisplatin concentrations of 0.125 and 0.25 μM. $*$, $p < 0.01$; $**$, $p < 0.001$; $***$, $p < 0.0001$.

through the purinergic receptor. We examined the mRNA expression of P2 receptors as well as downstream components of the pathway that are correlated with anticancer activity in CG3 cells. The RT-PCR results show that CG3 cells express the $P2Y_1$, Y_4, and Y_{11} types of G protein-coupled purinergic receptors (Fig. 2A). The possible routes after receptor binding to ATP (or clodronate) are the generation of Ca^{2+} signaling from ER Ca^{2+} through activation of phospholipase C (PLC)-IP_3 or the increase of cAMP/PKA by activated adenylyl cyclase. When ATP or clodronate was locally applied to CG3 cells, a large ATP- or clodronate-induced Ca^{2+} tran-

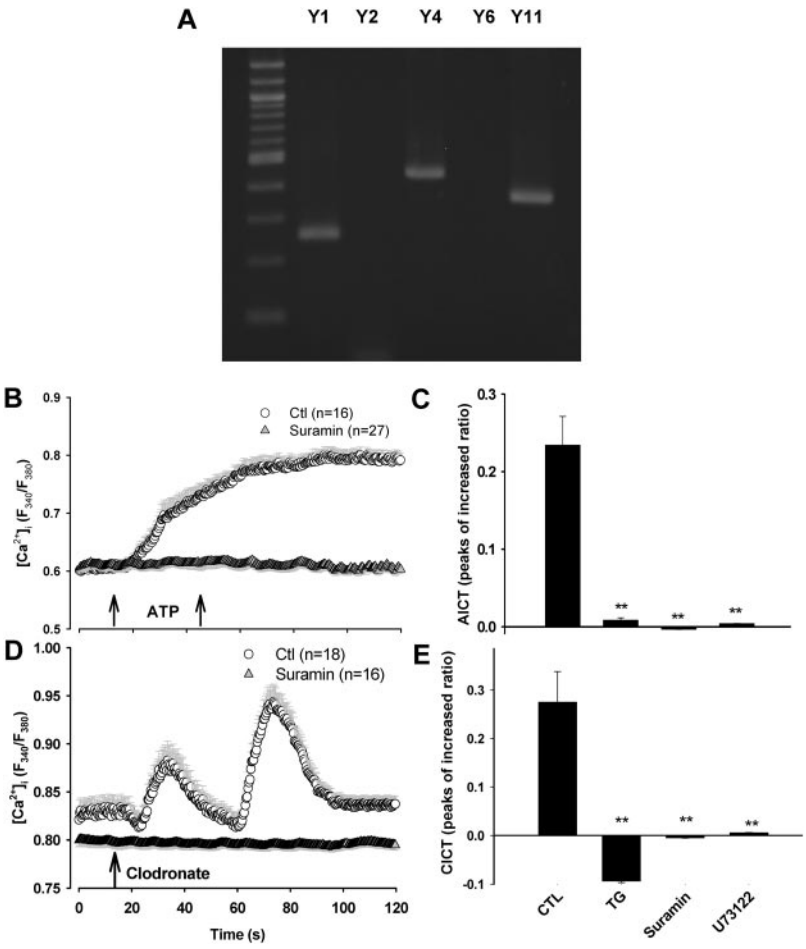

Fig. 2. ATP and clodronate induce elevated Ca^{2+} through the purinergic P2 receptor cascade. A, RT-PCR results from CG3 cells indicated that these cells expressed P2 purinergic receptors, including $P2Y_1$, Y_4, and Y_{11}. B to E, time-lapse changes of cytosolic Ca^{2+} was seen when ATP (B) or clodronate (D) was transiently applied as indicated by the arrow(s). C and E, statistical data for the ATP/clodronate-induced Ca^{2+} response under various antagonists, e.g., ER Ca^{2+} pump blocker TG, P2Y receptor antagonist suramin, and PLC inhibitor U73122 is shown, respectively.

sient was generated (Fig. 2, B and D). Suramin completely inhibited all the ATP/clodronate-induced responses in both CG3 (▲ in Fig. 2, B and D) and PTC cells (data not shown). The ER Ca^{2+} ATPase inhibitor thapsigargin (TG), the P2 antagonist suramin, and a PLC blocker (U73122) all abolished the ATP/clodronate-induced responses (Fig. 2, C and E). A unique store-operated Ca^{2+} entry (SOCE)-like activity (Ca^{2+} wave) was also observed (Fig. 2D).

The ER Ca^{2+} sensor D1ER was transfected into CG3 cells (Fig. 3A) to measure the dynamic changes of ER Ca^{2+} during ATP/clodronate-induced responses (Fig. 3, C and D). It was found that ER Ca^{2+} levels decreased when ATP or clodronate was applied (solid lines in Fig. 3, C and D), and this decrease was abolished by preloading with PLC inhibitor U73122 (dotted lines in Fig. 3, C and D). Together, these results confirmed that in CG3 cells, the ER releases Ca^{2+} in response to ATP or clodronate treatment and that a P2Y-PLC-Ca^{2+} signaling cascade exists to respond to extracellular ATP and clodronate.

Close Contact between ER and Mitochondria Functionally Affects on Mitochondrial Membrane Potential through Clodronate-Induced Ca^{2+} Signaling. It is known that mitochondria can uptake Ca^{2+} when the ER store is released; therefore, mitochondria could be one of the targets of ATP or clodronate. We visualized the subcellular localization and distribution of mitochondria and ER using confocal microscopy (Fig. 4, A and B). We also assayed mitochondrial functions transfecting mitochondrial Ca^{2+} sensor RP-mito or staining of TMRE to monitor the dynamic changes of $[Ca^{2+}]_{MT}$ (Fig. 4C) or changes of Ψ_M (Fig. 4, D and E), respectively. It was possible to clearly see the close contact between the ER and the mitochondria in both CG3 and PTC primary cultures (merged images in Fig. 4, Ad and Bd). Clodronate was shown to induce an increase in $[Ca^{2+}]_{MT}$ (Fig. 4C) as well as the depolarization of mitochondrial membrane potential Ψ_{MT} (Fig. 4, D and E). These results suggest that mitochondria can take up Ca^{2+} in response to the clodronate, which subsequently affects depolarization of Ψ_{MT}.

Clodronate Induces cAMP-Dependent Apoptotic Cell Death through a PLC- and SOCE-Independent Purinergic Pathway. We determined how clodronate induced the inhibition of cell proliferation. The detection of green fluorescent-tagged annexin V (Fig. 5A) or caspase-3 (Fig. 5C) by flow cytometry and the TUNEL assay (Fig. 6A) indicates that both ATP and clodronate induce CG3 apoptosis and that suramin can prevent this apoptotic effect (Fig. 5, A and C, right). In addition, the cAMP inhibitor SQ22536 rescued the clodronate-induced inhibition of CG3 cell proliferation (0.25–1 mM ATP; Fig. 6B, ♦). Neither the PLC antagonist U73122 (○ in Fig. 6B) nor the SOCE blocker 2-aminoethyl diphenylborinate (2-APB) (Fig. 6B, △) can reverse this anti-CG3 effect of clodronate. The clodronate-induced but not ATP-induced cell death was further enhanced by forskolin, which is an agonist of PKA (Fig. 6, C and D).

The summarized data on annexin V (Fig. 5B) and caspase-3 (Fig. 5D), together with the results of the TUNEL

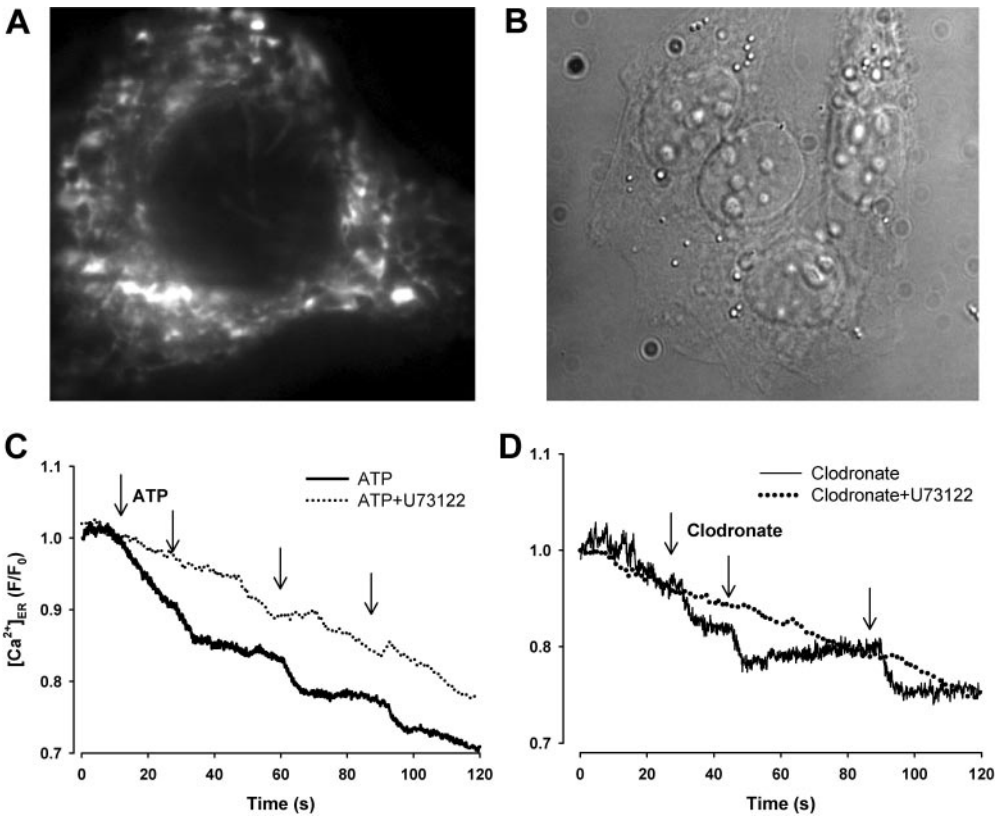

Fig. 3. ATP and clodronate induce ER Ca^{2+} release in CG3 cells. The representative fluorescent (D1ER-transfected) and transmitted images of CG3 cells are shown in A and B, respectively. The representative data from D1ER-transfected CG3 cells show that ATP (C) and clodronate (D) triggered (arrows) a decrease (solid lines) in D1ER intensity compared with the data from pretreatment with the PLC inhibitor U73122 (dotted lines).

and MTT assays in combination with several pharmacological blockers (Fig. 6B), suggest that clodronate induces CG3 cell apoptosis through the P2Y purinergic receptor-cAMP/PKA cascade but independently of PLC- and SOCE-Ca^{2+} signaling.

Inhibition of Thyroid Cancer Cell Growth by Clodronate. To further study the role of clodronate in the tumor malignancy of CG3 cells in vitro, migration/invasive and soft agar colony assays were used. Results showed that the in vitro migratory invasion (Fig. 7A) and colony formation (Fig. 7B) abilities of CG3 cells treated with clodronate were significantly decreased compared with those of nontreated CG3 cells (control; $p < 0.001$ in Fig. 7, A and B). Furthermore, the blocking effect of clodronate on cell migration and colony formation for the CG3 cells can be significantly reversed by adding the purinergic antagonist suramin ($p < 0.001$ in Fig. 7, A and B). The combination of clodronate (0.25 mM) with cisplatin (10 μM) enhanced the anti-PTC effect (Fig. 7C). Furthermore, the treatment effect of radiotherapy (2 Gy), chemotherapy (cisplatin), or a combination for CG3 cells can be significantly improved by the treatment with clodronate, and these effects can be further blocked by the treatment with suramin (Fig. 7D, $p < 0.001$). Thus, manipulation of P2 purinergic receptor signaling in clodronate-treated CG3 can effectively eliminate the migration ability of CG3 cells, suggesting that P2 signaling could be a therapeutic target to prevent metastasis of PTC.

Tumor Growth and Metastasis Are Inhibited by Clodronate in an Animal Model. We further investigated the antiproliferative and anticancer effects of clodronate in vivo. To measure the tumor volume and monitor the growth effects, CG3 cells were transfected by lentivector combined with GFP and followed by in vivo GFP imaging (Chen et al., 2008). The three groups of CG3-GFP-positive cells were individually injected into the neck region of nude mice (control, CG3-GFP only; CG3-GFP treated with clodronate; and CG3-GFP treated with clodronate plus suramin; for each group, $n = 8$ mice). The tumor volumes for CG3 treated with clodronate (20 mg/kg) were significantly decreased compared with those for CG3 alone or CG3 treated with clodronate (20 mg/kg) plus suramin (10 mg/kg; Fig. 8, A and B). Our data further showed that the clodronate-mediated antitumor or therapeutic effect could be significantly blocked by the treatment with suramin (Fig. 8, A and B). There was no additional decrease in tumor volumes in CG3 cells treated with clodronate plus SQ22563 and suramin (data not shown). During the 4-week follow-up, the body weights of these three groups of mice were not significantly decreased (Fig. 8C). However, there were significant differences between the control group (CG3 cells alone without any treatment) and the study group (CG3 treated with clodronate) in the fourth week follow-up ($p < 0.05$; Fig. 8C). In contrast, the changes of body weight in the control and the CG3 treated with clodronate plus suramin groups were slightly increased (Fig. 8C). Taken together, our data confirm that clodronate may have therapeutic potential for PTC treatment in vivo and indicate that the activation of P2 purinergic receptor signaling was partially involved in the antitumor effect of clodronate.

Discussion

In this study, we demonstrated first that treatment with clodronate can directly inhibit the proliferation and tumorigenicity of papillary types of thyroid cancer cells in vitro and in vivo (Figs. 1, 7, and 8). Second, suramin, the P2 receptor antagonist, is not only able to block all the clodronate-in-

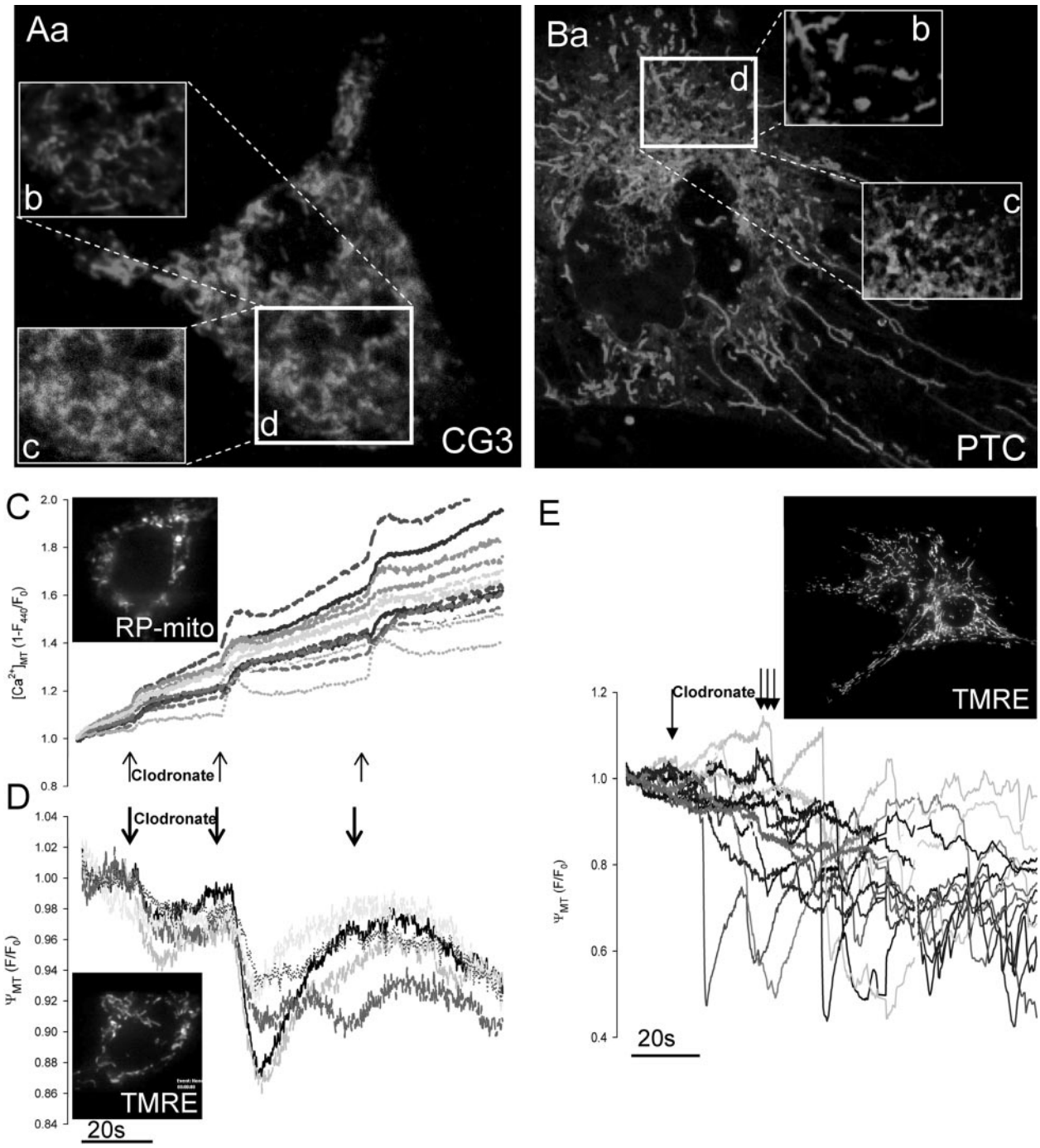

Fig. 4. Colocalization of the ER and mitochondria and effects of clodronate on $[Ca^{2+}]_{MT}$ and Ψ_{MT}. Close contact between mitochondria (b) and ER (c) under confocal laser scabbing microscopy is shown in CG3 (A) and PTC (B). Mitochondria were fluorescent tagged through transfection of pDsRed2-mito (Ab) or staining with TMRE (Bb), and ER was targeted through transfection of D1ER (Bc) or ER-Tracker Green (Bc). The fine structure and close contact region between the mitochondria and ER are shown in the merged images at high magnification (Ad and Bd). $[Ca^{2+}]_{MT}$ was monitored through transfection of mitochondrial targeted Ca^{2+} sensor RP-mito, and results are shown in C. Ψ_{MT} was dynamically recorded through staining with TMRE, and results are shown in D (CG3) and E (PTC). The increase of $[Ca^{2+}]_{MT}$ and decrease inΨ_{MT} induced by clodronate indicate the uptake of cytosolic Ca^{2+} by mitochondria, which subsequently cause the depolarization of mitochondrial membrane potential.

duced Ca^{2+} transients in treated PTC cells (Fig. 2) but also it effectively reverses the antitumor effect of clodronate in vitro as well as in vivo in xenotransplanted animals (Figs. 1, 7, and 8). However, clodronate-induced Ca^{2+} cascade including the PLC-ER Ca^{2+} release and SOCE was surprisingly unrelated to the molecular mechanism of clodronate-induced apoptosis in PTC cells (Fig. 6). Last, our data indicate that the cAMP/PKA cascade is involved in clodronate-induced CG3 cell apoptosis. This study is the first to report that the ATP-P2Y purinergic receptor signaling pathway plays a vital role in the antiproliferation effect of clodronate on PTC cells.

Anticancer Effects of Bisphosphonates and Clodronate. The BPs consist of nitrogen-containing BPs (N-BPs) and non-N-BPs and are analogs of endogenous pyrophosphates. They have been specifically used for more than three decades as an antibone disease agent to prevent the resorp-

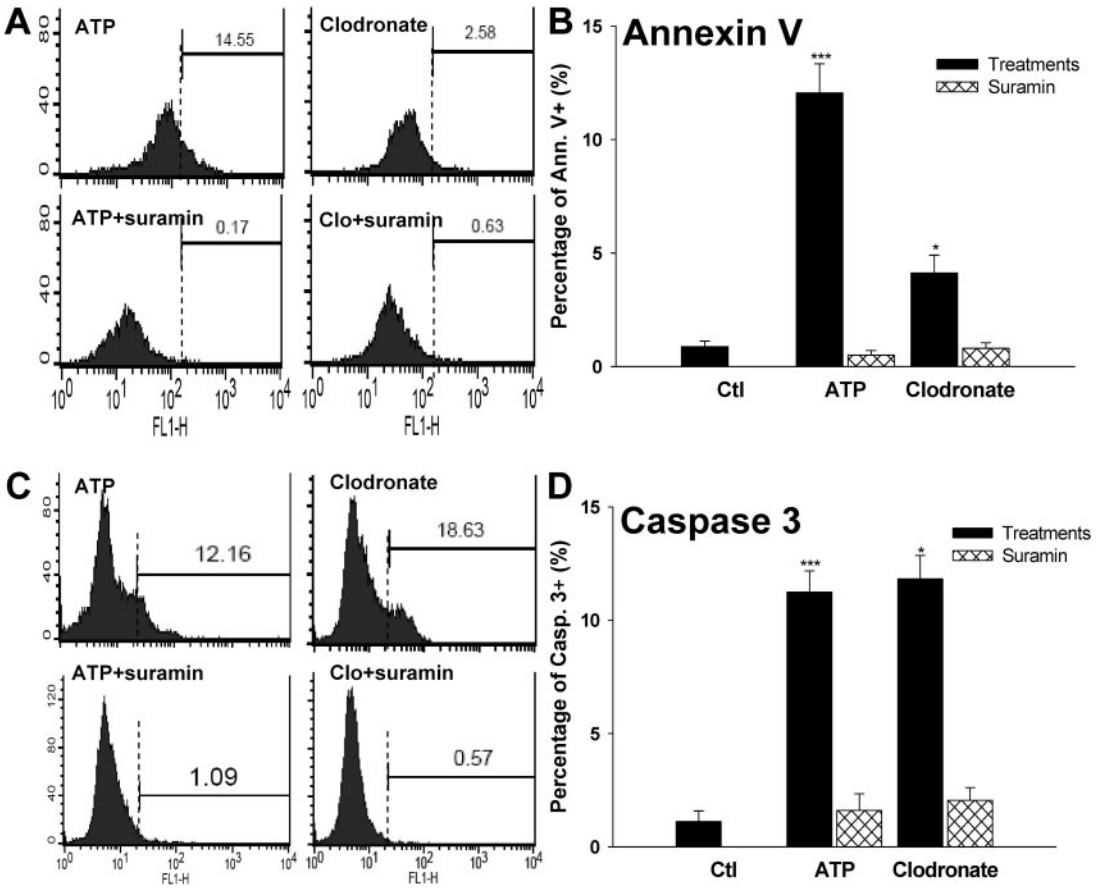

Fig. 5. Clodronate induces cell apoptosis through the purinergic pathway. A, representative flow cytometric results show that ATP (14.55%) or clodronate (2.58%) induced a large population of annexin V (FL1-H)-stained cells. These apoptotic events were blocked by cotreatment with suramin (200 μM; 0.17% in ATP + suramin and 0.63% in clodronate + suramin). C, both ATP and clodronate induced an increase of caspase-3 in CG3 cells. The summarized data of annexin V and caspase-3 are shown in B and D, respectively.

tion of bone by normal osteoclasts (Lehenkari et al., 2002). In contrast, oral administration of non-N-BP clodronate was first reported in 1998 to reduce the onset of bone metastasis in breast cancer patients (Diel et al., 1998). After a 10-year-long term evaluation, Diel's group further demonstrated that clodronate exhibits the capabilities of reducing the incidence of new bony and visceral metastasis in women with breast cancer and improves the overall survival in primary breast cancer patients with micrometastases to the bone marrow (Diel et al., 2008).

Furthermore, BPs are also known to induce growth arrest or cell apoptosis in breast (Brown et al., 2004), prostate (Brown et al., 2004), and thyroid (Yang et al., 2004; this study) tumors in which bone metastasis happens frequently (Green, 2003). The question of what mechanism underlies the apoptotic effect of BPs on cancer cells has been investigated for years (Green, 2003). It has been suggested that both types of BPs, clodronate (non-N-BP) and alendronate (N-BP), exert unique antiproliferation signals on noncancer osteoclasts or macrophages through internalization/endocytosis (the cytosolic entry), metabolite formation, and the targeting of specific pathways (Lehenkari et al., 2002). Very recently, this entry-metabolite-apoptosis mechanism has also been shown in some cancer cells (Mönkkönen et al., 2008). However, the degree of BP entry/uptake into cells to form certain amounts of BP metabolites varies with cell types. Sometimes BP entry/uptake requires a combination with liposomes to facilitate endocytosis events (Lehenkari et al., 2002; Mönkkönen et al., 2008). This study reveals that the ATP-P2 receptor signaling pathway exists for the clodronate-induced anticancer effect to help mediate apoptotic signaling.

Effect of ATP on Cancer Cells through Various Types of Purinergic Receptors. Several isoforms of P2Y receptors are known to be expressed in a broad range of normal tissues to functionally and physiologically respond to extracellular ATP or related nucleotides (White and Burnstock, 2006). Some examples include the stimulation of expression and release of interleukin-6 in epidermal keratinocytes (Inoue et al., 2007), improvement of cardiac contraction in mouse cardiomyocytes ($P2Y_{11}$) (Balogh et al., 2005), prevention of apoptosis in human neutrophils ($P2Y_1$ and Y_{11}) (Vaughan et al., 2007), and regulation of rat hepatocyte function ($P2Y_1$, Y_{12}, and Y_{13}) (Dixon et al., 2004).

Alternatively, ATP has also been reported to have a specific role on cancer cells, e.g., to attenuate cancer cell growth and to cause cancer cell death (White and Burnstock, 2006). This unique anticancer ability of ATP has been found in many types of cancers, such as human melanoma ($P2Y_1$) (White et al., 2005), intestinal epithelial carcinoma ($P2Y_1$, Y_4, and Y_2) (Coutinho-Silva et al., 2005), prostate cancer ($P2Y_{11}$) (Shabbir et al., 2008a), bladder cancer ($P2Y_{11}$) (Shabbir et al., 2008b), and thyroid cancer in this study ($P2Y_1$, Y_4, and Y_{11} might be involved; Fig. 2A).

Although it has been noticed that certain P2Y receptor

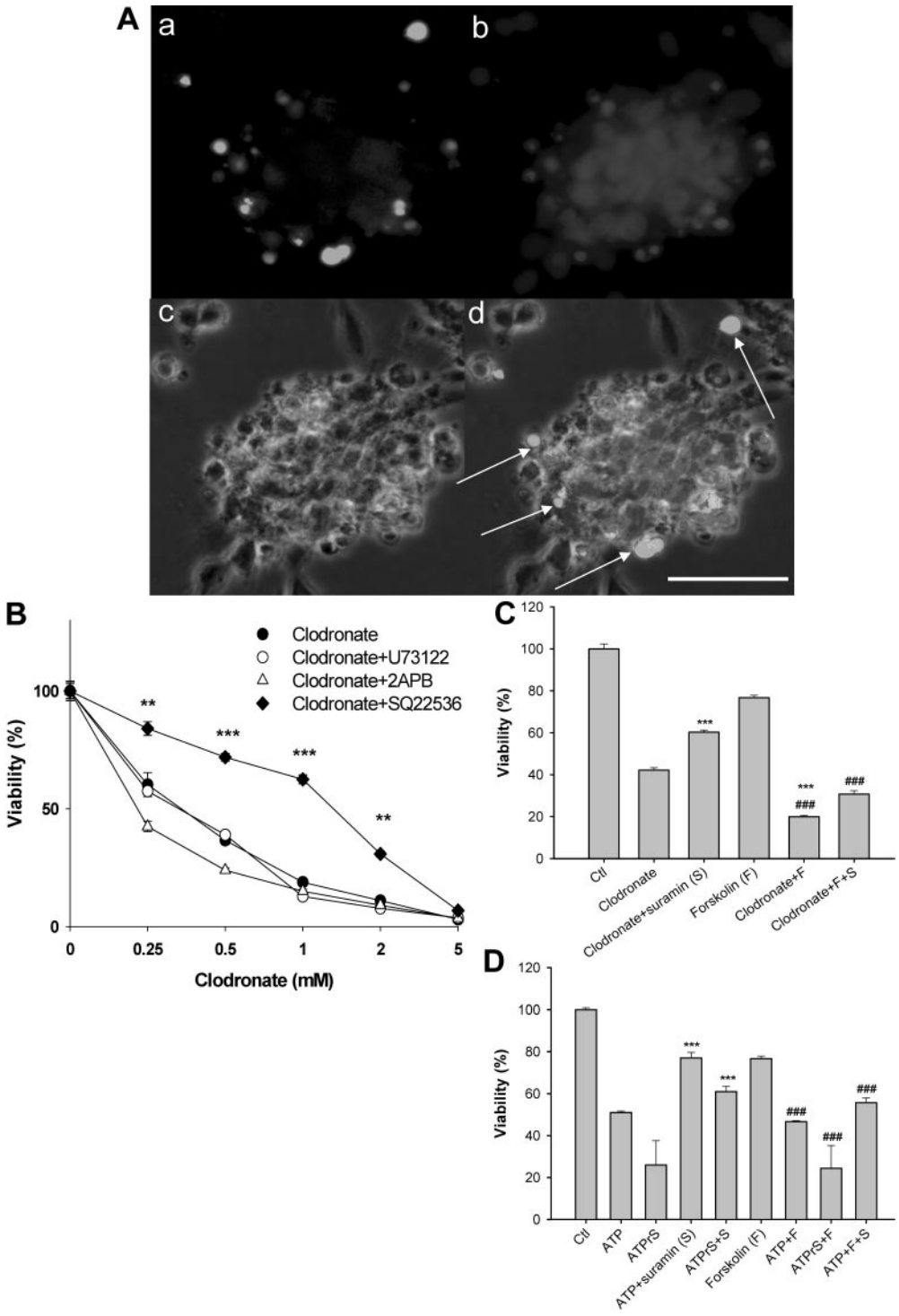

Fig. 6. Clodronate induces cAMP-dependent cell apoptosis. A, representative images from a TUNEL assay under treatment of clodronate are shown. The TUNEL and nucleus stains, and bright-field photos are shown in a to c, respectively (scale bar, 50 μm). B, neither the PLC antagonist U73122 nor SOCE blocker 2-APB could prevent the clodronate-induced cell proliferation inhibition (○ and △). In contrast, cAMP reversed this clodronate-induced cell proliferation inhibition (♦). C, clodronate-induced cell death (1 mM after a 48-h treatment) was enhanced by forskolin but not blocked by the P2 antagonist suramin. D, ATP and adenosine-5′-*O*-(3-thio)triphosphate (ATPγS)-induced CG3 cell death (1 mM after a 48-h treatment) was not enhanced by forskolin. ***, CG3 treated with clodronate ± forskolin (ATP or ATPγS in D) + suramin (S) versus CG3 treated with clodronate (ATP or ATPγS in D) only; ###, CG3 treated with clodronate + forskolin ± suramin versus CG3 treated with forskolin; $p < 0.0001$.

signaling may govern cancer cell growth, the mechanism is still highly controversial (Mamedova et al., 2006; White and Burnstock, 2006). Various isoforms of P2Y receptors and the diversity of downstream transduction mechanisms between different normal and cancerous tissues might explain the opposite effects of ATP, related compounds, or both. Examples of signal transduction mechanisms include the different G proteins, such as G_q, G_{11}, or G_s cascades; activation of PLC-ER Ca^{2+} release after the breakdown of phosphatidylinositol into IP_3; SOCE signaling triggered by ER Ca^{2+} release; and the cAMP/PKA pathway regulated by the activity of adenylate cyclase (White and Burnstock, 2006). Additional studies should be carried out to identify specific alternations of downstream cascades between cancer and normal cells to provide clues for the differential signaling and to understand how ATP induces apoptotic signals specifically within cancer cells (White and Burnstock, 2006).

cAMP Is Involved in the Clodronate-Induced Anti-PTC Signaling. Because the cAMP inhibitor SQ22563 attenuated the anti-CG3 effect of clodronate (Fig. 6C), a possible downstream mechanism for the anticancer property of ATP/clodronate might be involved in the increase of cAMP

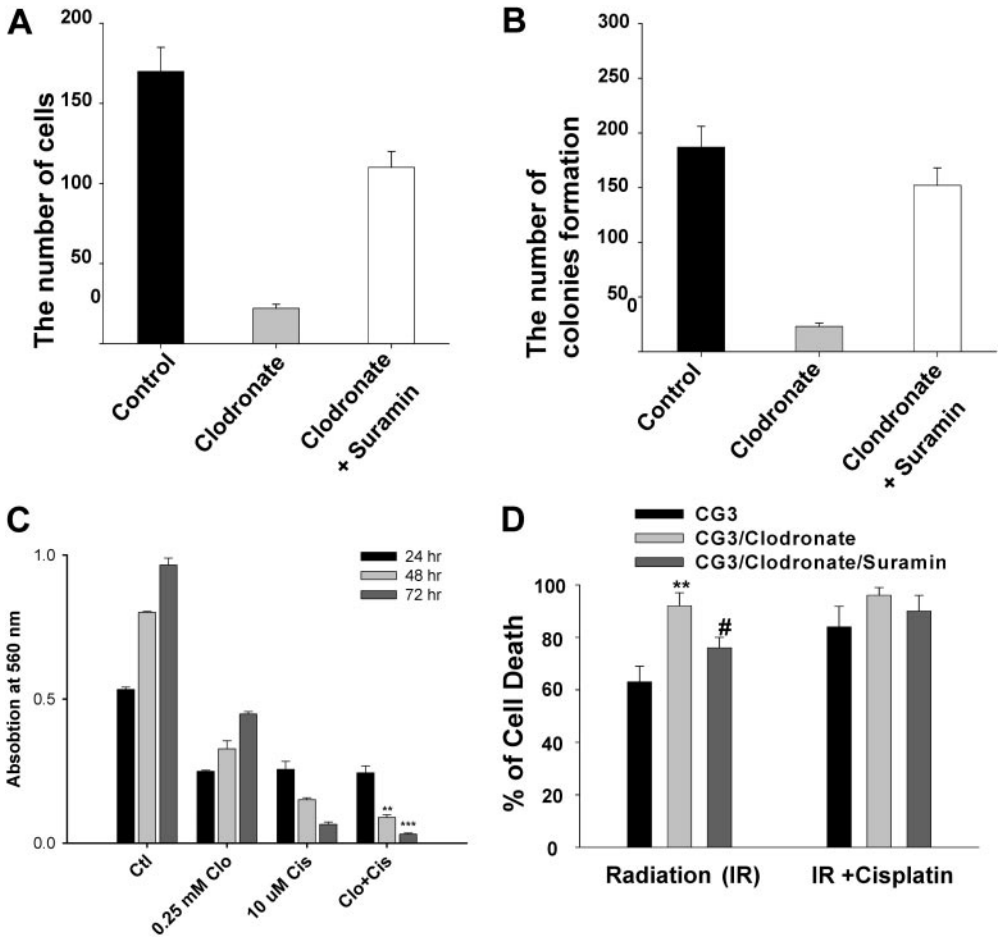

Fig. 7. Inhibition of tumorigenic capabilities in thyroid cancer by clodronate. The in vitro migration/invasive assay (A) and colony formation in soft agar (B) of CG3 cells were evaluated under clodronate treatments. Compared with nontreated CG3 cells (black bars in A and B), both the abilities of migration (gray bar in A) and colony formation (gray bar in B) were significantly decreased ($p < 0.001$). The purinergic antagonist suramin significantly reversed the blocking effect of clodronate on cell migration and colony formation ($p < 0.001$). C, MTT absorption at 560 nm of CG3 cells after 24-, 48-, and 72-h treatments with various combinations of drugs: 0.25 mM clodronate, 10 μM cisplatin, and 0.25 mM clodronate with 10 μM cisplatin. D, treatment of clodronate was shown to significantly improve the anticancer effect of radiotherapy (IR) (2 Gy) and/or chemotherapy (cisplatin) for CG3 cells (gray bar), and suramin abolished these recovering effects (**, CG3 treated with clodronate + IR versus CG3 treated with IR only; #, CG3 treated with clodronate + IR versus CG3 treated with clodronate + suramin + IR; $p < 0.001$).

through the activation of adenylyl cyclase after the ATP/clodronate-P2 receptor binding. It has recently been found that cAMP/PKA (Martinez-Velazquez et al., 2007) and the cancer progression-related molecular mitochondrial Smac/DIABLO (Martinez-Ruiz et al., 2008) are involved in cancer cell apoptotic signaling (Martinez-Velazquez et al., 2007). Furthermore, the report that altered expression of Smac and related molecules contributed to chemotherapy resistance in thyroid cancer cells (Tirrò et al., 2006) provides valuable information about the therapy for thyroid cancer. It would be interesting to further investigate whether Smac/DIABLO plays a role in clodronate-induced PTC apoptosis.

The Ca^{2+} Signaling after P2 Receptor Binding Is Not Related to the Anti-PTC Ability of Clodronate. The role of Ca^{2+} ions as the second messenger that exerts an anticancer effect could be mediated by extracellular Ca^{2+} influx through ionotropic P2X receptors, the ER Ca^{2+} release through the metabotropic P2Y receptors coupled with G protein-PLC to open ionotropic IP_3 receptor (Coutinho-Silva et al., 2005; White et al., 2005), or the further generation of SOCE.

In previous studies, normal human thyrocytes were shown to express certain types of P2Y receptors (Schöfl et al., 1995). SOCE activities were also reported in normal rat thyroid cell cultures (Marsigliante et al., 2002). The transformed and cancerous/undifferentiated thyrocytes were observed to have a disturbed or defective PLC-Ca^{2+} signaling process after activation of the purinergic cascade (Schöfl et al., 1997; Elia et al., 2003; Yang et al., 2004).

In this study, a Ca^{2+} waveform-like pattern of the clodronate-induced Ca^{2+} response in PTC cells was found (Fig. 2D). It was further observed that removal of the extracellular Ca^{2+} using EGTA or by adding an inhibitor of SOCE, 2-APB, would block this waveform and the subsequent second clodronate-induced Ca^{2+} response (data not shown). This information suggests that SOCE may be involved in the events that follow the clodronate-induced Ca^{2+} response. However, the results that U73122 and 2-APB are not able to prevent the clodronate-induced CG3 cell death (Fig. 6B) indicate that the anticancer effect of clodronate is not through PLC- and SOCE-related cascades.

ER-Mitochondria Relationship Might Play Some Role in the Clodronate-Induced Anti-PTC Cascade. Previous reports have suggested that the mitochondria of cancer cells could be a specific target of anticancer drugs (Holmuhamedov et al., 2002). Further studies have suggested that oxidative damage to the mitochondria such as depolarization of Ψ_{MT} could be an efficient strategy for killing cancer cells (Linford et al., 2006). In this study, mitochondrial depolarization (Fig. 4, D and E) was accompanied by an increase in $[Ca^{2+}]_{MT}$ (Fig. 4C), which has a functional role in buffering the cytosol from ER calcium release during the clodronate-induced Ca^{2+} response. A similar result was found in osteoclasts where clodronate and the proposed clodronate metabolite adenosine-5′-[β,γ-dichloromethylene]triphosphate also decreased the Ψ_{MT} (Lehenkari et al., 2002). Here, we alternatively suggest that the depolarization of the Ψ_{MT} occurs by the clodronate-induced Ca^{2+} response

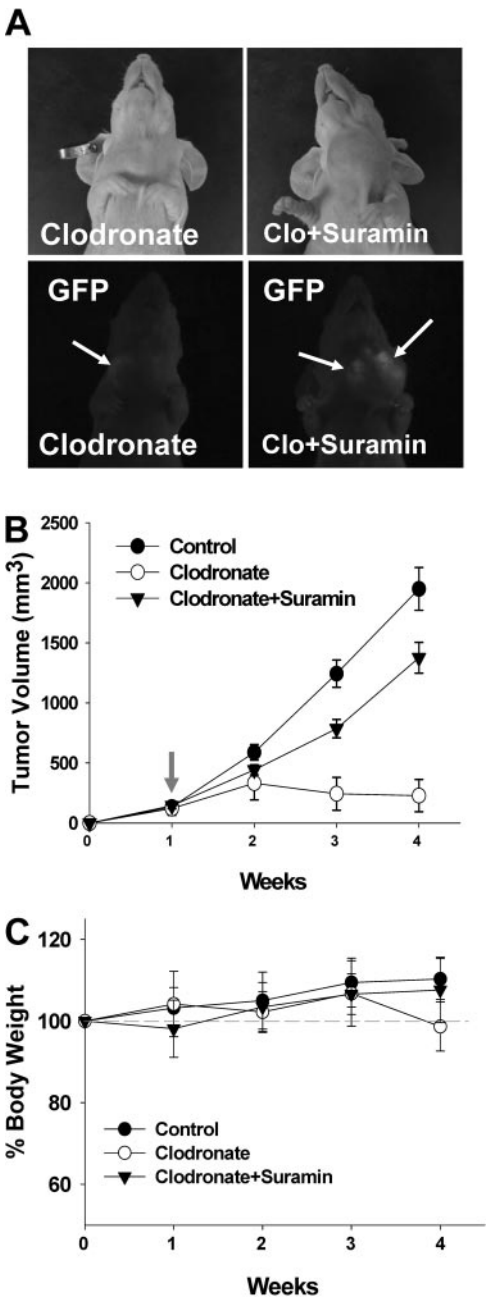

Fig. 8. Clodronate prevents thyroid cancer cell growth in xenotransplanted mice, and this effect was reversed by the purinergic antagonist suramin. A and B, CG3-treated tumor volumes were significantly decreased by clodronates (20 mg/kg) compared with those for CG3 alone or CG3 treated with clodronate (20 mg/kg) plus suramin (10 mg/kg). The treatment with suramin (10 mg/kg) could effectively block the antitumor effect of clodronate-treated xenotransplanted CG3 cells. Arrows in A, tumors. Arrow in B, beginning of clodronate alone treatment (20 mg/kg i.p., twice a week) or clodronate (20 mg/kg i.p., twice a week) plus suramin (10 mg/kg i.p., twice a week) treatment. C, change in percentage of body weight in the control group, CG3 treated with clodronate group, or CG3 treated with clodronate + suramin group was measured and recorded.

through an ER-mitochondria chain reaction (Fig. 4, Ad and Bd). Additional studies are warranted to confirm the relationship among mitochondrial depolarization, caspase-3-Smac/DIABLO, and cAMP/PKA signaling.

In conclusion, it was demonstrated here that clodronate could inhibit the proliferation and tumorigenicity of PTC in vitro and in vivo partially through activation of apoptosis. A P2Y receptor-cAMP cascade, possibly $P2Y_{11}$ signaling, was also identified to play a crucial role in clodronate-induced PTC apoptosis. The anticancer property and combined therapeutic benefit of clodronate in PTC and other tumors should be considered in translational oncology studies. Therefore, clodronate and other analogs/derivates might be a relevant adjuvant for the clinical treatment of papillary thyroid tumors refractory to radioiodine treatment.

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