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Regulation of Osteoclastogenesis by Gap Junction Communication

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Abstract Receptor activator of NF-κB ligand (RANKL) is crucial in osteoclastogenesis but signaling events involved in osteoclast differentiation are far from complete and other signals may play a role in osteoclastogenesis. A more direct pathway for cellular crosstalk is provided by gap junction intercellular channel, which allows adjacent cells to exchange second messengers, ions, and cellular metabolites. Here we have investigated the role of gap junction communication in osteoclastogenesis in mouse bone marrow cultures. Immunoreactive sites for the gap junction protein connexin 43 (Cx43) were detected in the marrow stromal cells and in mature osteoclasts. Carbenoxolone (CBX) functionally blocked gap junction communication as demonstrated by a scrape loading Lucifer Yellow dye transfer technique. CBX caused a dose-dependent inhibition (significant ≥ 90 μM) of the number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells formed in 7- to 8-day marrow cultures stimulated by parathyroid hormone (PTH; 10 nM) or forskolin (FSK; 1 μM). Furthermore, CBX (100 μM) significantly inhibited prostaglandin E_2 (PGE_2; 10 μM) and 1,25$(OH)_2$-vitamin D_3 stimulated osteoclast differentiation in the mouse bone marrow cultures. Consequently, quantitative real-time polymerase chain reaction (PCR) analysis demonstrated that CBX downregulated the expression of osteoclast phenotypic markers, but without having any significant effects on RANK, RANKL, and osteoprotegerin (OPG) mRNA expression. However, the results demonstrated that CBX significantly inhibits RANKL-stimulated (100 ng/ml) osteoclastogenesis in the mouse bone marrow cultures. Taken together, our results suggests that gap junctional diffusion of messenger molecules interacts with signaling pathways downstream RANKL in osteoclast differentiation. Further studies are required to define the precise mechanisms and molecular targets involved. J. Cell. Biochem. 99: 528–537, 2006.

Key words: carbenoxolone; connexin 43; gap junction; osteoclast; RANKL

Osteoclasts are multinucleated, highly specialized, bone resorbing cells that originate from hematopoietic precursor cells of the monocytic/macrophage lineage. Several studies have provided evidence that cellular crosstalk and interactions between osteoblasts/stromal cells and osteoclast precursors play a central role in osteoclastogenesis and osteoclast function. Receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) are factors crucial in osteoclast differentiation and activation produced by osteoblasts/stromal cells in response to different pro-resorptive and calciotropic factors [Tsukii et al., 1998]. Osteoblasts/stromal cells also produce osteoprotegerin (OPG), a soluble decoy receptor for RANKL that inhibits both differentiation and function of osteoclasts. RANKL signaling is mediated by cytoplasmic adaptor proteins and downstream activation of different protein kinases and transcription factors [see review Boyle et al., 2003]. Although a crucial role of the RANKL signaling pathway in osteoclastogenesis has clearly been demonstrated, crosstalk with other intercellular transduction pathways may be important in the differentiation process. Gap junctional channels transmit signals between adjacent cells in almost all tissues and are of importance in diverse biological

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processes, including coordination between cells and cell differentiation. The channels are formed by juxtaposition of two hemi channels, allowing direct intercellular communication via diffusion of ions, metabolites, and small soluble molecules (up to 1 kDa in size), including cAMP, Ca^{2+}, ATP, and inositol (1,4,5)-triphosphate [Yeager et al., 1998]. The gap junction channels are formed by hexameric connexin proteins (Cx). Around 20 highly homologous products of Cx genes have been identified in humans and rodents. A single cell type may express one or several Cx, which form channels with different permeability and ionic selectivity [Evans and Martin, 2002; Boyle et al., 2003]. Cx43 is the most widely spread and best studied of the Cx family, with much attention being focused on its regulation. Phosphorylation is an important regulatory mechanism of gap junctional communication and may interact with the trafficking, assembly/disassembly, degradation or gating of gap junction channels [Lampe and Lau, 2004].

The major gap junction protein expressed in osteoblasts, Cx43, is associated with expression of genes critical for bone formation and phenotypic markers of mature osteoblasts. [Chiba et al., 1994; Schiller et al., 1997, 2001; Lecanda et al., 2000; Furlan et al., 2001]. Parathyroid hormone (PTH) regulates gap junction coupling and communication in osteoblastic cell cultures, an effect partly mediated through an increase in cAMP production [Schiller et al., 1992, 1997, 2001; Chiba et al., 1994; Civitelli et al., 1998; Lecanda et al., 2000; Furlan et al., 2001]. Thus, several studies indicate that intercellular communication via gap junctions is crucial in coordinating cellular responses to external signals and promoting osteoblast differentiation. However, our knowledge of gap junction intercellular communication in osteoclast differentiation and function is fragmentary. Immunohistochemical studies have demonstrated Cx43 immunoreactive sites in osteoclasts [Jones et al., 1993; Su et al., 1997; Ilvesaro et al., 2000]. We have also reported, in a recent study, Cx43 mRNA expression in mouse bone marrow cultures and in micro isolated osteoclasts [Ransjo et al., 2003] and we and others have shown that gap junction inhibitors decrease bone resorption in vitro [Ilvesaro et al., 2000; Ransjo et al., 2003] suggesting that gap junctional channels mediate signals important in the bone resorption process although the exact mechanism is still unclear. In the present study, we have addressed the question whether gap junction channels mediate signals in osteoclast differentiation and whether there is an interaction with the RANK/RANKL signaling pathway.

MATERIALS AND METHODS

All animal protocols were approved by the Animal Care and Use Committe of the University of Umeå.

Mouse Bone Marrow Cultures

Mouse bone marrow cells were isolated and cultured essentially as previously described. Briefly, 6-to 9-week-old CsA mice were killed by cervical dislocation. The tibiae and femur were removed and dissected free from adhering soft tissues. The bone ends were cut off and the marrow cavity was flushed with α-modification of Minimum Essential Medium (α-MEM; Life Technologies, Paisley, Scotland), supplemented with 10% heat-inactivated fetal calf serum (FCS; Life Technologies), and antibiotics. The marrow cells were thereafter collected into tubes, washed, and cultured in 35-mm Nunclon culture dishes (2×10^6 cells/cm^2) or in 24-well plates (Nunc, Denmark, 1×10^6 cells/cm^2), each well containing a Thermanox coverslip (Nunc, Naperville, IL). Cells were cultured in α-MEM medium supplemented with 10% FCS, L-glutamic acid, and antibiotics and incubated at 37°C in 5% CO_2 in a humified tissue culture incubator. After a 24-h attachment period in control medium, culture medium was replaced and cells were cultured in medium with or without addition of a stimulator of osteoclast formation; synthetic bovine PTH (10 nM; PTH 1-34; Bachem, Switzerland,) or forskolin (FSK; 1 μM; Calbiochem, La Jolla, CA), recombinant murine RANKL and recombinant human TNFα were from R&D systems, Abingdon, UK. Prostaglandin E_2 (PGE_2) was from Upjohn Co., Kalamazoo, MI. 1,25$(OH)_2$-vitamin D_3 was generously provided by Hoffman-La Roche, Basel, Switzerland. PGE_2, 1,25$(OH)_2$-vitamin D_3, and FSK were dissolved in ethanol; the final concentration never exceeded 0.1%. Cultures were fed with fresh medium every 3 days. Carbenoxolone (CBX; Sigma Chemical Co., St. Louis, MO; prepared as a fresh 10-mM stock solution in medium) was added at stated concentrations to cultures after 3 days.

Staining and Counting of Osteoclast-Like Cells

After culture for 8–9 days, Thermanox coverslips were washed in Tyrode's salt solution, fixed with citrate-acetone-formaldehyde for 30 s, dried at room temperature, and thereafter stained for tartrate-resistant acid phosphatase (TRAP) activity, using a commercially available staining kit (Sigma Chemical Co.). Staining for TRAP was performed according to the instructions supplied by the manufacturer, except for staining time which was reduced to 20 min. Dark red cells containing three or more nuclei were counted as osteoclasts. Multinucleated TRAP-positive cells formed in the mouse bone marrow cultures show phenotypic markers for differentiated osteoclasts, such as CT-receptors and capacity to excavate resorption lacunae in slices of bone [Ransjo et al., 1999].

Immunostaining for Cx43

Bone marrow cells grown in 35-mm culture dish for 8–9 days in the presence of PTH were rinsed carefully with phosphate-buffered saline (PBS) thereafter fixed in ice-cold methanol/acetate (50:50; vol/vol) for 5 min, washed in PBS, and further incubated for 15 min, at room temperature, with 2% inactivated swine serum (DAKO A/S, Glostrup, Denmark) in PBS-added Triton X-100 (final concentration 0.5%, Merck Eurolabs, Darmstadt, Germany). The samples were incubated for 45 min at room temperature with a 1:400 dilution of rabbit polyclonal anti-Cx43 (Zymed Co., CA) and after 3×5 min washes with PBS, a 1:500 dilution of FITC-conjugated swine anti-rabbit IgG (DAKO A/S) was added for 45 min at room temperature, washed 3×5 min with PBS and kept dark at 4°C until photomicrographs were taken (Leica fluorescence microscope; Leica Microsystems, Wetzlar, Germany).

Scrape Loading/Dye Transfer Assay

Scrape loading dye transfer (SL/DT) is a fast and simple technique to assess gap junction intercellular communication [el-Fouly et al., 1987]. For the SL/DT assay, we utilized bone marrow cell cultures grown in 35-mm culture dish for 8–9 days in the presence of PTH (10 nM) and with or without the addition of CBX (120 μM). The cells were rinsed three times carefully with PBS. An aliquot of 2 ml of PBS containing 0.05% Lucifer Yellow (Molecular Probes, Eugene, OR) was added and a scrape was made on the monolayer using a surgical blade. The cells were incubated for 3 min at room temperature in the dye solution and then rinsed 3×5 min with PBS. The cells were then fixed with 1 ml of 4% paraformaldehyde. The spreading of Lucifer Yellow through gap junctions was monitored and light and fluorescence micrographs taken after fixation of the cells.

RNA Extraction and First-Strand Complementary DNA Synthesis

Mouse bone marrow cells used for RT-polymerase chain reaction (PCR) analysis were cultured for 8–9 days in 35-mm Nunclon culture dishes (Nunc) with the stated additions of test substances. Medium was removed and after washing with PBS, total RNA was extracted with TRIZOL Reagent (GIBCO BRL, Life Technologies) following the manufacturer's protocol. The purity and quantity of RNA was determined spectrophotometrically and the integrity of the RNA preparations was examined by gel electrophoresis. Extracted total RNA was treated with deoxyribonuclease I (Ambion, Intermedica, Sweden) and thereafter 1 μg of total RNA was reverse transcribed into single-stranded cDNA using AMV Reverse Transcriptase, oligo-p-$(dT)_{15}$ (Life Technologies), and a 1st Strand cDNA Synthesis Kit (Roche, Diagnostics Corp., Indianapolis, IN) following the manufacturers' instructions. The cDNA was kept at −20°C until used for PCR.

Quantitative Real-Time PCR

RNA was isolated and cDNA produced as described above. Quantitative PCR analysis was performed on an ABI PRISM 7900 HT Sequence Detection System and software (Applied Biosystems, Foster City, CA) using cDNA diluted 1/20 in nuclease-free water, the TaqMan Universal PCR Master Mix kit (Applied Biosystems, Warrington, UK), 300–900 nM of each primer and 100–150 nM for the labeled probes (reporter fluorescent dye VIC at the 5′-end and quencher fluorescent dye TAMRA at the 3′-end; Applied Biosystems, UK). Sequences for primers and probes and the Genbank accession number are shown in Table I. The relative target mRNA expression was computed from target C_t values and β-actin C_t values using the standard curve method (Applied Biosystems).

TABLE I. Primer and Probe Sequences, GenBank Accession no. for Quantitative Real-Time PCR Assay

Primer and probe sequences (5′ to 3′)	GenBank no.
β-actin	
GGACCTGACGGACTACCTCATG	M12481
TCTTTGATGTCACGCACGATTT	
CCTGACCGAGCGTGGCTACAGCTTC	
CTR	
AGTTGCCCTCTTATGAAGGAGAAG	NM007588
GGAGTGTCGTCCCAGCACAT	
TCTGTACTGCAACCGCACCTGGGA	
Cath K	
ATATGTGGGCCAGGATGAAAGTT	NM007802
TCGTTCCCCACAGGAATCTCT	
CCACGGCAAAGGCAGCTAAATGCA	
TRAP	
CGACCATTGTTAGCCACATACG	BC01960
TCGTCCTGAAGATACTGCAGGTT	
CACTGCCTACCTGTGTGGACATGA	
Cx43	
TTTGACTTCAGCCTCCAAGGA	NM63801
GTCTGGGCACCTCTCTTTCACT	
ACTTTGGCGTGCCGGCTTCACTT	
RANKL	
TGGAAGGCTCATGGTTGGAT	AF053713
CATTGATGGTGAGGTGTGCAA	
AGGCTTGCCTCGCTGGGCCAC	
OPG	
AGCTGCTGAAGCTGTGGAA	U94331
GGTTCGAGTGGCCGAGAT	
CCAAGACATTGACCTCTGTGAAAGCA	
RANK	
TGCCTACAGCATGGGCT	AF019046
AGAGATGAACGTGGAGTTACTGTTT	
CCAGTGAAGCAGCAGCCAGCAT	

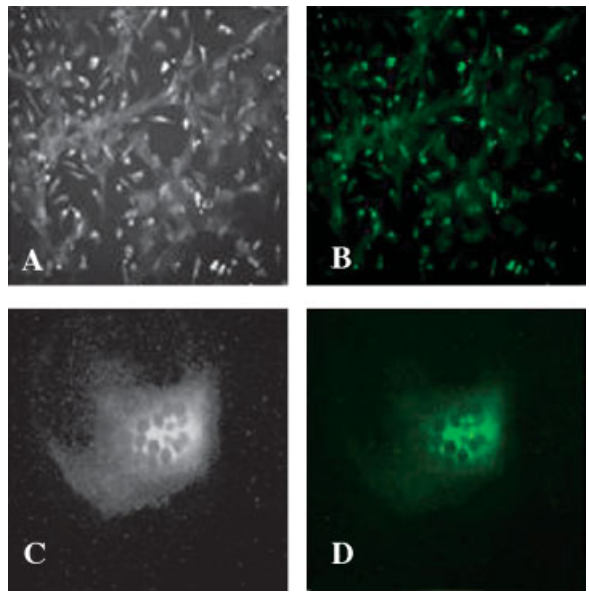

Fig. 1. A–D: Immunohistochemical demonstration of connexin 43 (Cx43) protein in stromal cells (A, B) and osteoclasts in mouse bone marrow cultures (C, D). Mouse bone marrow cells were grown for 8–9 days in the presence of parathyroid hormone (PTH; 10 nM). Cells were fixed and stained with anti-Cx43 as described in Materials and Methods. Photomicrographs were taken from the mouse bone marrow cultures and displays Cx43 protein staining in stromal cells (A, B) and in osteoclasts (C, D). Light micrographs are displayed in (A, C) and corresponding fluorescence micrographs in (B, D). Original magnification 150–200×.

Statistical Analysis

All experiments were repeated at least three times. The data were analyzed by SPSS statistical software. To test the difference between groups and within the groups, one-way ANOVA and Bonferroni post-hoc tests were performed, respectively. A P-value of less than 0.05 was considered significant. Data are presented as mean ± SEM.

RESULTS

Mouse bone marrow cultures were employed to study the effect of gap junction blockage on the recruitment of multinucleated osteoclasts. We have earlier reported that TRAP-positive osteoclast-like cells form in these marrow cultures in response to PTH or FSK and the osteoclasts express mRNA for Cx43 [Ransjo et al., 1999, 2003]. In the present study, we localized Cx43 protein in the bone marrow cultures by immunohistochemistry. Abundant Cx43 immunoreactivity was seen in the marrow stromal cells displayed as unevenly distributed spots in the cells (Fig. 1A,B). Immunoreactive staining was demonstrated in many osteoclasts and mostly seen in areas around the nuclei (Fig. 1C,D). To further investigate involvement of gap junction intercellular signaling in osteoclastogenesis, we used a hemisuccinate derivative of 18beta-glycyrrhetinic acid; CBX, a water-soluble gap junction blocker [Davidson et al., 1986]. To verify that CBX is a blocker of functional gap junctional coupling in our cultures, we performed SL/DT experiments in confluent bone marrow cultures. A scrape line was made in the cell culture (Fig. 2A). Lucifer Yellow dye was thereafter added to the culture and the transfer of dye from cells in the scrape line to adjacent cells was monitored. In some cultures, cells at the scrape line was retracting away from the scrape line (see Fig. 2A–C), however, this did not influence the spreading of dye. In cultures stimulated with PTH (10 nM), dye was transferred through several cells at both sides of the scrape line (Fig. 2B,C). In contrast, minimal transfer of dye of cells in the scrape line to adjacent cells was seen

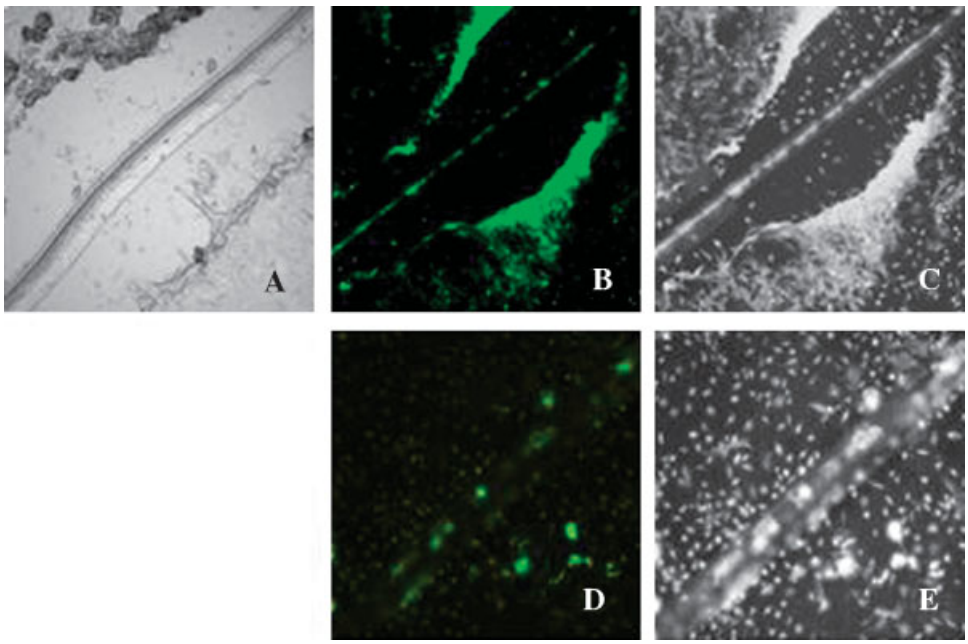

Fig. 2. **A–E**: Scrape load dye transfer in mouse bone marrow cultures. Mouse bone marrow cells were grown for 8–9 days in the presence of PTH (10 nM) and without (B, C) or with (D, E) the addition of carbenoxolone (CBX) (120 μM). Lucifer Yellow was added and a scrape line was made, cells were incubated and then fixed as described in Materials and Methods. Phase contrast micrograph of cell culture and a scrape line is demonstrated in (A). The spreading of Lucifer Yellow through gap junctions was monitored and photomicrographs taken after fixation of the cells. Light (C, E) and fluorescence (B, D) micrographs. Original magnification 100×.

in PTH-stimulated cultures treated with CBX (120 μM; Fig. 2D,E). Concluding that CBX worked as a functional blocker of gap junctions in our cell cultures, we thereafter studied the effect of CBX on the formation of TRAP-positive multinucleated osteoclast-like cells in stimulated marrow cultures. From early experiments, we could conclude that presence of CBX during the initial stage of cultures reduced the total number of attached marrow cells (data not shown). Therefore, in the following series of experiments, CBX was added to culture media after 3 days of culture. A clear inhibitory effect of CBX (120 μM) on the number of TRAP-positive multinucleated cells formed in FSK (1 μM) and PTH (10 nM) stimulated marrow cultures is demonstrated (Fig. 3). Dose-response experiments demonstrated a concentration-dependent inhibitory activity of CBX over a rather short concentration range. Significant inhibition of stimulated osteoclast formation was obtained at and above 90 μM CBX ($P \leq 0.005$) and a complete inhibition of osteoclast formation seen at 120 μM (a representative experiment demonstrated in Fig. 4A). CBX (100 μM) could also block osteoclastogenesis induced by PGE_2 (1 μM) and $1,25(OH)_2$-vitamin D_3 (10 nM, Fig. 4B). We next used quantitative real-time PCR and analyzed gene expression in marrow cultures stimulated with PTH or FSK for 8 days and with or without addition of 120 μM of CBX after day 3. We wanted to confirm the inhibitory effects of CBX on TRAP-positive multinucleated cells with effects on osteoclast-phenotypic genes; Cathepsin K, TRAP, calcitonin receptor (CTR); gene specific primer sequences shown in Table I. PTH and FSK caused a significant upregulation of Cx43 (Fig. 5A) and the specific osteoclast marker CTR (Fig. 5B) and Cathepsin K gene (Fig. 5C) expression ($P < 0.05$) as compared to control cultures. A clear upregulation of the TRAP gene expression in response to PTH and FSK was also observed (Fig. 5D). Moreover, the expression of RANK (Fig. 5E) and RANKL (Fig. 5F) were upregulated and a decreased expression of OPG was seen in response to FSK and PTH (Fig. 5G). CBX had no significant effects on gene expression in control cultures although a decrease in OPG expression is demonstrated (Fig. 5G). In accordance to the inhibitory effects on TRAP-positive multinucleated cells, CBX caused significant ($P < 0.05$) downregulation of CTR (Fig. 5B), Cathepsin K (Fig. 5C), and Cx43

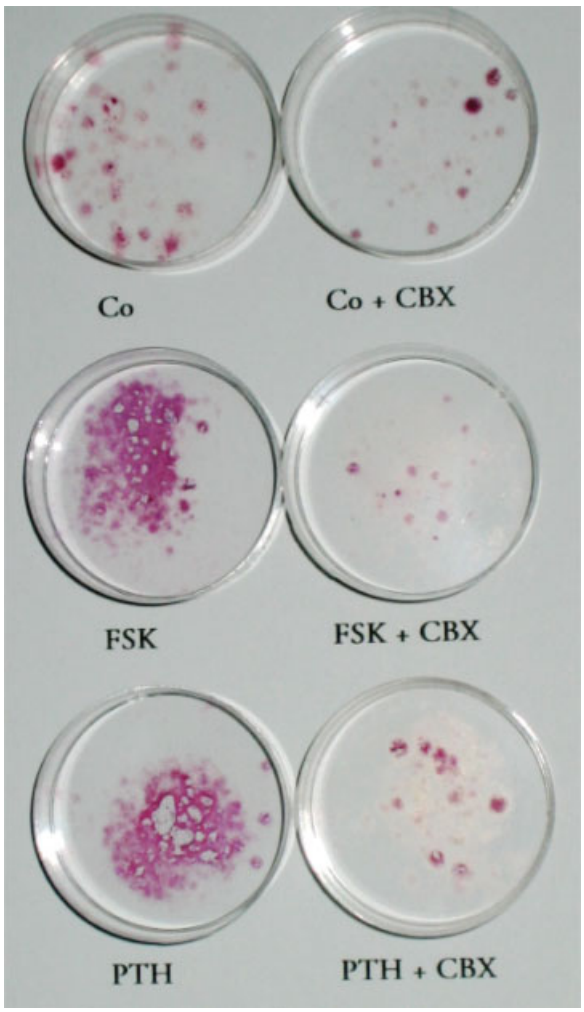

Fig. 3. Effect of CBX on PTH- and forskolin (FSK)-stimulated mouse bone marrow cells. Mouse bone marrow cells were grown for 8–9 days with or without PTH (10 nM) or FSK (1 μM) and without or with addition of CBX (120 μM) after 3 days of culture. Cultures were fixed and stained for tartrate-resistant acid phosphatase (TRAP) as described in Materials and Methods.

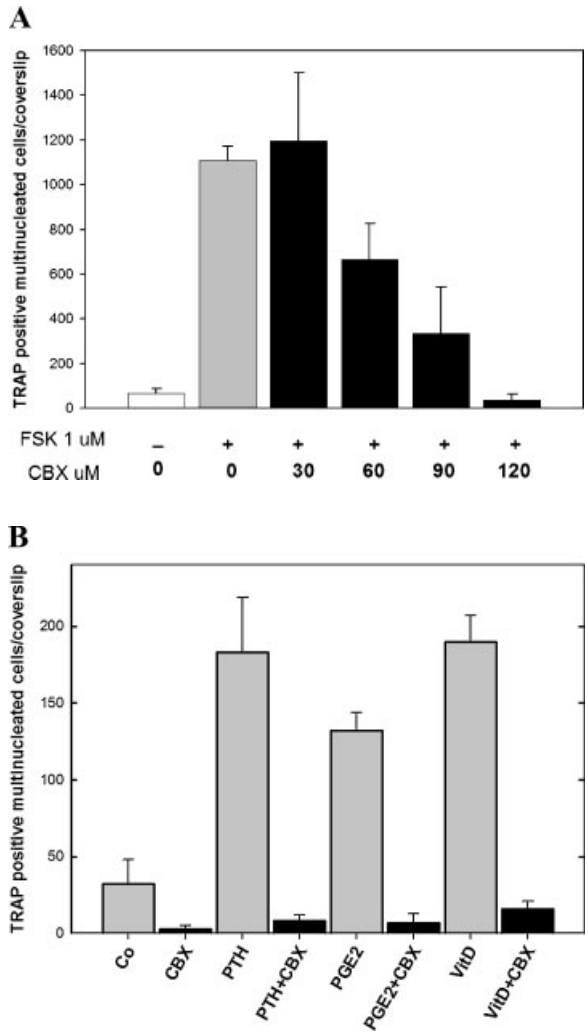

Fig. 4. **A**, **B**: Effects of CBX on the number of TRAP-positive multinucleated cells formed in FSK-, PTH-, prostaglandin E_2 (PGE_2)-, and 1,25 $(OH)_2$-vitamin D_3-stimulated mouse bone marrow cultures. Mouse bone marrow cultures were grown for 8 days with or without the addition of FSK (A; 1 μM) or PTH (B; 10 nM), PGE_2 (B; 1 μM) and 1,25$(OH)_2$-vitamin D_3 (B; 10 nM) in the absence or presence of different concentrations of CBX (A; 30–120 μM and B; 100 μM) added after 3 days of culture. Cells were fixed and stained for TRAP as described in Materials and Methods. Dark red cells containing three or more nuclei were counted as osteoclasts. Data represent mean ± SEM. CBX caused a significant inhibition of FSK-stimulated osteoclast formation ≥90 μM (A; $P \leq 0.005$). A significant inhibition of PTH-, PGE_2-, and 1,25$(OH)_2$-vitamin D_3-stimulated osteoclastogenesis is demonstrated in (B) ($P \leq 0.05$).

(Fig. 5A) gene expression in the FSK and PTH-stimulated cultures. However, CBX did not have any significant effects on the expression of RANK (Fig. 5E), RANKL (Fig. 5F), or OPG (Fig. 5G) in PTH- or FSK-stimulated cultures. In similarity, PGE_2 (1 μM) and 1,25$(OH)_2$-vitamin D_3 (10 nM) significantly increased CTR, Cathepsin, K and RANKL mRNA expression and CBX (100 μM) caused a significant inhibition of osteoclast phenotypic markers but not RANKL expression (data not shown). In summary, CBX inhibits osteoclast differentiation induced by agonists who all increased RANKL but without having any effect on the expression of RANKL. We next asked whether the effect of CBX in osteoclastogenesis in mouse bone marrow cultures is down stream RANK–RANKL interactions. As shown in Figure 6A–C, CBX (100 μM) significantly inhibited the number of TRAP-positive multinucleated cells formed in marrow cultures stimulated with RANKL (100 ng/ml). In line with this, CBX inhibited RANKL-induced

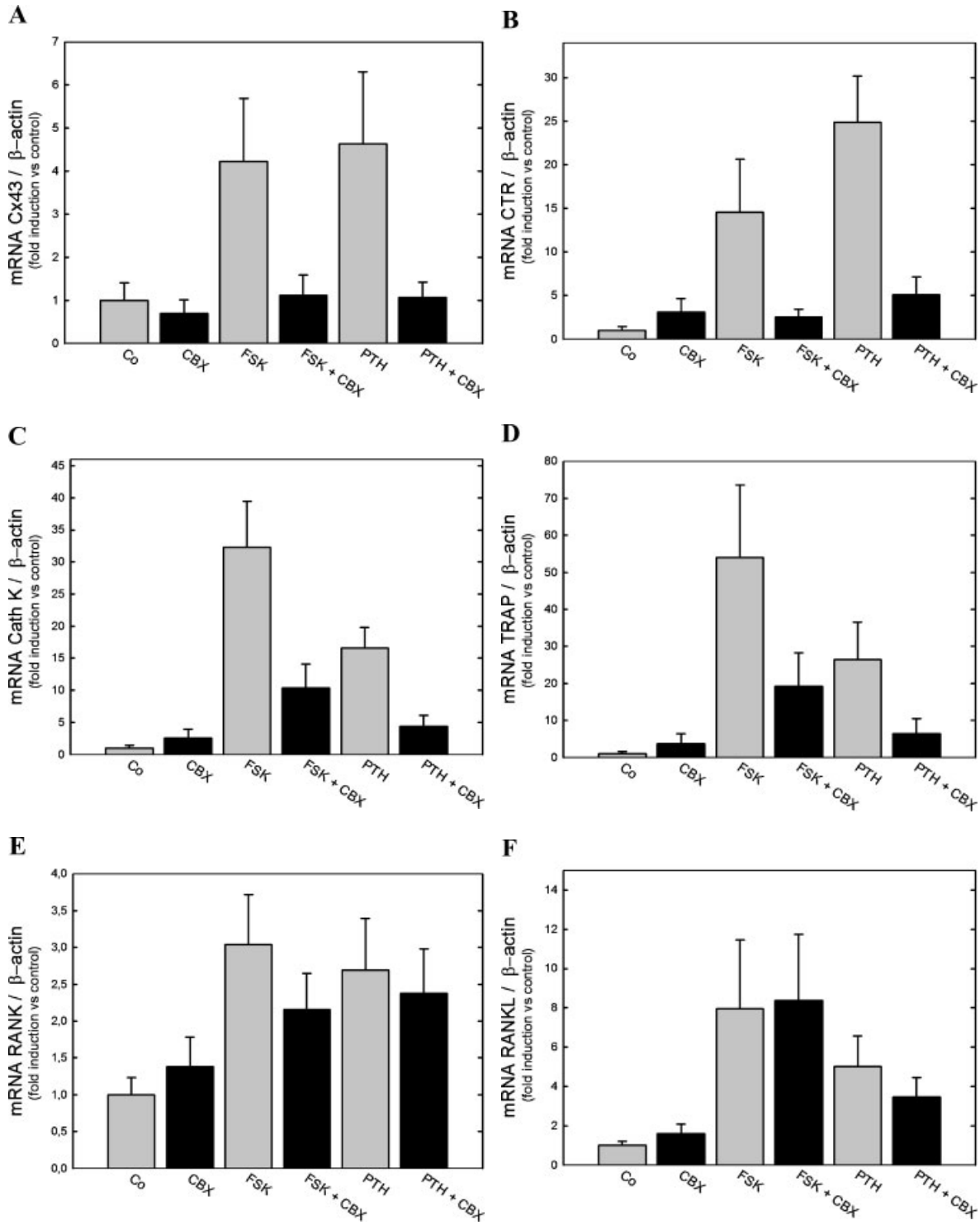

Fig. 5. **A**–**G**: Effect of CBX on Cx43, calcitonin receptor (CTR), Cathepsin K, TRAP, osteoprotegerin (OPG), RANK, and receptor activator of NF-κB ligand (RANKL) mRNA expression in PTH- or FSK-stimulated mouse bone marrow cultures. Mouse bone marrow cells were cultured for 8–9 days with or without FSK (1 μM) or PTH (10 nm) and in the absence or presence of CBX (120 μM) added after 3 days of culture. Quantitative real-time polymerase chain reaction (PCR) analysis was performed as described in Materials and Methods. Values represent specific gene expression from 4 to 5 experiments normalized versus β-actin and compared to control cultures. Data represent mean ± SEM. Effects of PTH and FSK on (**A**) Cx43, (**B**) CTR, and (**C**) Cathepsin K gene expression are significant ($P < 0.05$) as compared to control cultures. Effect of CBX on (A) Cx43, (B) CTR, and (C) Cathepsin K gene expression in the FSK- and PTH-stimulated cultures are significant as compared to corresponding stimulated cultures without CBX ($P < 0.05$).

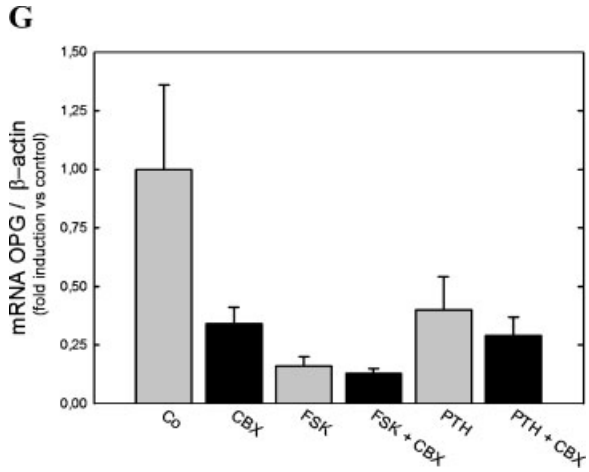

Fig. 5. (*Continued*)

gene expression of the osteoclast phenotypic markers CTR, Cath K, and TRAP (data not shown).

DISCUSSION

As far as we know, this is the first study that demonstrates the critical importance of gap junction-mediated signals in osteoclast differentiation. Earlier studies have demonstrated Cx43 in marrow stromal cells and suggest that stromal Cx43-mediated intercellular communication plays a role in the supportive ability for hemopoietic progenitors and stem cells [Cancelas et al., 2000; Durig et al., 2000]. In the present study, we demonstrate Cx43 mRNA and protein expression in mouse bone marrow stromal cells and we confirm our and Ilvesaro's earlier studies which demonstrated Cx43 in murine osteoclasts [Ilvesaro et al., 2000; Ransjo et al., 2003]. We did not try to quantify the Cx43 protein expression in marrow cultures since the antibody we used recognizes both phosphorylated and non-phosphorylated forms of the protein. It is also difficult to decide whether the immunostaining is localized in channels or diffused in the cytoplasm. We used a pharmacological non-toxic blocker of gap junction, CBX, to study the function and regulation of gap junction communication in osteoclast recruitment. The mechanism of CBX as a gap junction inhibitor is not clear but it has been proposed that CBX intercalates into the plasma membrane and binds to the gap junction connexons, inducing a conformational change which results in closure of the channels [Davidson et al., 1986; Davidson and Baumgarten, 1988]. Using a scrape loading Lucifer Yellow dye transfer assay, we established that CBX is an efficient blocker of intercellular communication in the marrow cultures. We have also confirmed the non-toxic inhibitory effect of CBX on gap junction intercellular communication in the marrow cultures using flow cytometry and parachuting dye transfer techniques (data not shown; manuscript in preparation). Our dose-response curve for the inhibitory effect of CBX in osteoclast recruitment corresponds with the dose-response curve for CBX inhibition in gap junction communication that has been demonstrated earlier in other in vitro systems [Davidson et al., 1986; Davidson and Baumgarten, 1988; Bani-Yaghoub et al., 1999]. However, the concentration-effect curve for CBX can vary a little depending on serum proteins, which may explain effects at lower concentrations of CBX in some experiments [Davidson et al., 1986; Davidson and Baumgarten, 1988]. When CBX was added during the first day of culture, cells lost attachment, which might be explained by an interactive effect on early cell adhesion. The cell adhesion molecule N-cadherin appears to be associated with cell contacts responsible for the presence and/or maintenance of Cx43-mediated gap junctional communication in osteoblasts/stromal cells [Rundus et al., 1998; Marie, 2002; Nagahata et al., 2004]. When CBX is added after 3 days of culture, no loss of cell attachment could be detected. CBX must be present during the remaining culture period for maximum inhibitory effect on osteoclast differentiation (data not shown). This may be explained by the fact that the blocking effect of CBX is demonstrated to be rapidly reversible [Davidson et al., 1986; Davidson and Baumgarten, 1988]. The present study shows that CBX $\geq$ 90 μM significantly inhibits the number of TRAP-positive multinucleated cells and expression of osteoclast-phenotypic genes. This suggests that signals/molecules transferred through gap junction channels are critical in agonist-stimulated osteoclast differentiation in the mouse bone marrow culture system. It is demonstrated that gap junction communication regulates gene expression in osteoblasts [Chiba et al., 1994; Schiller et al., 1997, 2001; Lecanda et al., 2000; Furlan et al., 2001], and a crucial question is if the inhibitory effect of CBX on osteoclastogenesis is secondary to effects on osteoblasts/stromal cells. It has been

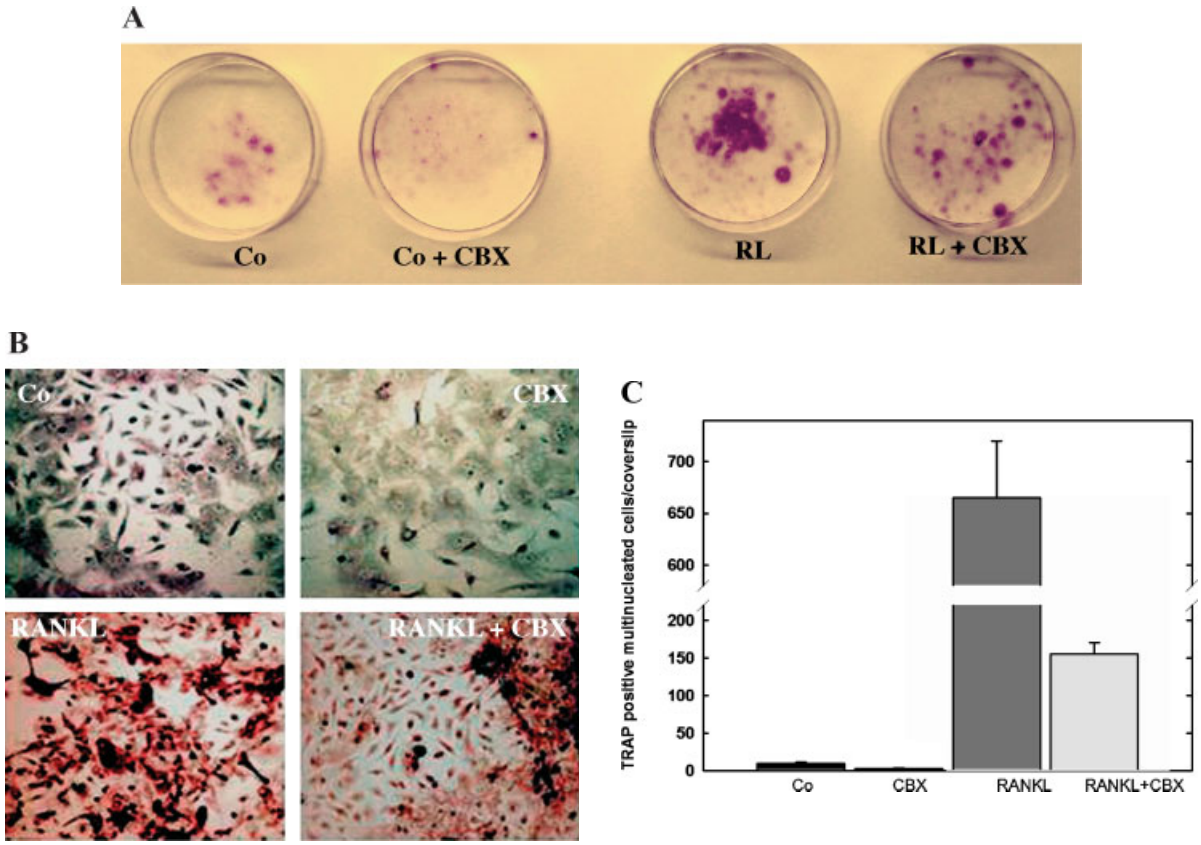

Fig. 6. **A–C**: Effect of CBX on RANKL-stimulated mouse bone marrow cells. Mouse bone marrow cells were grown for 8–9 days with or without RANKL (100 ng/ml) and without or with the addition of CBX (100 μM) after 3 days of culture. Cells were fixed and stained for TRAP as described in Materials and Methods. Dark red cells containing three or more nuclei were counted as osteoclasts. Data represent mean ± SEM.

demonstrated that PTH enhance gap junctional communication in osteoblastic/stromal cells presumably through cAMP [Civitelli et al., 1998] and cAMP/protein kinase A mediates regulation of osteoclastogenesis and expression of RANKL and OPG mRNAs by the marrow stromal cells [Lee et al., 1999; Kondo et al., 2002]. However, our observation that CBX has no effect on RANKL or OPG mRNA expression does not support that gap junction signals directly regulates stromal cells/osteoblast production of molecules in the RANK/RANKL signaling pathway. Another option is that CBX blocks signals essential for the differentiation and fusion of osteoclasts precursors but downstream RANK/RANKL. To address this issue, we looked for the effects of CBX on RANKL-stimulated osteoclastogenesis in marrow cultures and our results clearly demonstrate that CBX inhibits RANKL-stimulated differentiation of multinucleated TRAP-positive cells. Since CBX is added 3 days after the addition of RANKL, the effect of CBX is mainly on the terminal differentiation and fusion of osteoclasts. Thus, this indicates that of gap junction mediated signals acting downstream the RANK–RANKL interaction in osteoclast precursors and is crucial mediating differentiation and fusion of osteoclast precursors. We are currently investigating the precise mechanisms by which gap junctional communication regulates differentiation of osteoclasts and the molecular targets involved.

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