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Endothelin1-Induced Ca^{2+} Mobilization Is Altered in Calvarial Osteoblastic Cells of $Cx43^{+/-}$ Mice

Graziello Geneau · Norah Defamie · Marc Mesnil · Laurent Cronier

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Abstract During bone remodeling, osteoblastic (OB) cells have a central role leading to the production of extracellular matrix and its subsequent mineralization. As revealed by human physiopathologies, the OB differentiation process is essential for the control of calcium metabolism and normal bone formation. Moreover, accumulating data in the field of bone development suggest that connexin 43 (Cx43)-mediated gap junctional communication plays an important role in OB differentiation and function. Since Ca^{2+} has a central role in OB physiology, the aim of the present study was to investigate the hypothetical involvement of Cx43 in OB calcium homeostasis. We performed measurements of intracellular calcium activity ($[Ca^{2+}]_i$) by a cytofluorimetric method using Fluo-4 as a calcium indicator and endothelin-1 (ET-1) as a physiological calcium-mobilizing factor on cultured OB cells isolated from calvaria of $Cx43^{+/-}$ and $Cx43^{+/+}$ mice. Partial deletion of the Cx43 gene induced a significant decrease in the $[Ca^{2+}]_i$ rise elicited by ET-1. This reduction was not correlated to a decrease or a modification of ET receptor subtype expression as assessed by real-time reverse-transcription polymerase chain reaction. Pharmacological investigations led us to demonstrate that the significant difference in $[Ca^{2+}]_i$ peak amplitude during the ET-1 action was associated with decreased calcium influx involving L-type voltage-sensitive calcium channels, whereas calcium release from intracellular stores and implication of phospholipase C were not affected by the reduced expression of Cx43. In conclusion, our data demonstrate for the first time that the Cx43 level of expression and/or function is able to modulate the $[Ca^{2+}]_i$ mobilization in OB cells.

Keywords $[Ca^{2+}]_i$ · Connexin43 · Endothelin · Osteoblast · Transgenic mouse · Calcium channel

G. Geneau · N. Defamie · M. Mesnil · L. Cronier (✉)
Institut de Physiologie et de Biologie Cellulaires, CNRS UMR6187, Université de Poitiers, 40 avenue du recteur Pineau, 86022 Poitiers, France
e-mail: Laurent.Cronier@univ-poitiers.fr

Introduction

In addition to its supportive and protective functions, bone tissue is a dynamic system intimately associated with mineral metabolism of the body, mainly phosphocalcic. Bone turnover is a tightly coordinated process due to activation and function of different cell types under the control of transcriptional, growth and systemic factors (Baylink, Finkelman & Mohan, 1993; Yang & Karsenty, 2002; Provot & Schipani, 2005). During such a temporally and spatially regulated remodeling cycle, bone-forming osteoblastic (OB) cells, derived from mesenchymal stem cells, play a central role leading to the production and maturation of extracellular matrix and its subsequent mineralization by hydroxyapatite deposition on collagen. Then, the OB differentiation process is an essential event for the control of calcium metabolism and normal bone formation and remodeling. Differentiating OB cells sequentially acquire an expression profile from type I collagen, alkaline phosphatase (early criteria) to osteocalcin (late criterion) genes, which are required for matrix mineralization (Aubin et al., 1995).

Present in the mineral deposits, calcium is involved in bone physiology, and differentiating OB cells are crucial for the control of calcium metabolism. Furthermore, Ca^{2+} is required in multiple cellular functions that include secretion, ionic conductance, cell cycle regulation and programmed cell death (Berridge, Lipp & Bootman, 2000);

and defective regulation of Ca^{2+} signaling is cytotoxic (Clapham, 1995). Mechanisms inducing a rise of calcium activity in OB cells following exposure to various biologically active substances have been investigated in numerous *in vitro* studies and corresponded both to release from intracellular stores and calcium influx through plasma membrane (Lieberherr, 1987; Caffrey & Farach-Carson, 1989; Takuwa et al., 1989; Stern et al., 1995). The latter phenomenon involves activation of voltage-sensitive calcium channels (L- or T-type) as well as capacitive calcium entry (Yamaguchi et al., 1989; Duncan, Akanbi & Farach-Carson, 1998; Bergh et al., 2006).

The osteogenic process is highly coordinated and seems to involve direct intercellular communication mediated by the presence of gap junctions between OB cells. Indeed, much evidence has demonstrated that the predominant form of gap junction protein in OB cells, connexin43 (Cx43), was functionally related to the differentiation state of osteogenic cells (Schiller et al., 2001; Upham et al., 2003). During *in vitro* studies, any alteration of Cx43 expression affected the differentiation or mineralization process (Lecanda et al., 1998; Civitelli et al., 1998; Li et al., 2006). Moreover, targeted ablation of Cx43 gene in mice ($Cx43^{-/-}$) clearly demonstrated the link between Cx43 expression and OB differentiation with profound mineralization defects in elements of the skull as well as axial and appendicular skeleton (Lecanda et al., 2000). Recently, a progressive osteopenia assessed by bone mineral density and histomorphometric analyses was also detected in conditionally osteoblast-deleted Col/Cre;$Cx43^{-/fl}$ mice together with a defective OB function and an attenuated anabolic response to parathyroid hormone (PTH) (Chung et al., 2006). Similarly, in a temperature-sensitive human cell line (hFOB1.19), we have demonstrated that Cx43 expression level could influence the inhibitory action of endothelin-1 (ET-1) on differentiation of human OB cells (Niger et al., 2007).

Owing to the central role of Ca^{2+} in OB physiology and in the transduction pathways of PTH and ET-1, the principal aim of the present study was to investigate a hypothetic role of Cx43 on OB calcium homeostasis. ET-1 was then used as a physiological calcium mobilizing factor. To overcome the postnatal lethality of $Cx43^{-/-}$ mice (Reaume et al., 1995), we performed measurements of intracellular calcium activity ($[Ca^{2+}]_i$) by a cytofluorimetric method on cultured OB cells which were isolated from both $Cx43^{+/-}$ and $Cx43^{+/+}$ calvaria.

Partial deletion of the Cx43 gene led to reduced expression and function of the gap junctional protein as demonstrated by Western blot and gap-fluorescence recovery after photobleaching (FRAP) analyses and induced a significant decrease in the $[Ca^{2+}]_i$ rise elicited by ET-1. This reduction in calcium mobilization was not correlated to a decrease or a modification of ET A and/or B subtype expression as assessed by real-time reverse-transcription polymerase chain reaction (RT-PCR). Moreover, neither the liberation from calcium stores nor the transduction via phospholipase C or inositol triphosphate receptors was altered in $Cx43^{+/-}$ OB cells compared to control cells. Finally, our pharmacological studies using nonspecific (Ni^{2+} or SK&F96365) or specific (nifedipine) channel blockers provided evidence that the L-type voltage-sensitive calcium channels (L-VSCCs) implicated in the ET-1 response were specifically affected by the reduced expression of Cx43 in OB cells, leading to altered calcium influx.

Materials and Methods

Transgenic Mice

OB cells were extracted from calvaria of 3-day-old genetically modified newborn mice ($Gja1^{tm1Kdr}$; Jackson Laboratory, Bar Harbor, ME) and their origin's strain mice. Mutation is due to the insertion in-frame into exon 2 of Cx43 (*Gja1*) gene of a promoterless neomycin (Neo) gene (Reaume et al., 1995). As previously described, $Gja1^{tm1Kdr}$ homozygous ($Cx43^{-/-}$) mice die at birth due to a severe heart defect (Reaume et al., 1995). Consequently, only heterozygous ($Cx43^{+/-}$) and wild-type ($Cx43^{+/+}$) mice were used for the study. Tails from such mice were used for genotyping, and calvaria were kept in Dulbecco's minimum essential medium (DMEM) supplemented with penicillin (100 UI/ml) and streptomycin (100 μg/ml) at 4°C during the genotyping process. Genomic DNA was extracted in Tris-HCl buffer 50 mM (pH 8.0) containing 10 mM Na_2 ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate (SDS) and 2 mg/ml proteinase K (Roche Diagnostics, Mannheim, Germany) for 2 h at 65°C. DNA was precipitated with a mix containing ethanol 95% and 143 mM sodium acetate and rinsed in a 70% ethanol solution. DNA was then resuspended in a Tris-EDTA buffer for 45 min at 65°C and used as a template for PCR analysis. Cx43 and Neo sequences were amplified with 50 mIU/μl *Taq* polymerase (BioTaq Bioline; AbCys, Paris, France), and four primers were used: mCx43-forward (NM_010288), 5′-CCC-CAC-TCT-CAC-CTA-TGT-CTC-C-3′; mCx43-reverse, 5′-ACT-TTT-GCC-GCC-TAG-CTA-TCC-C-3′; mNeo-forward, 5′-GGC-CAC-AGT-CGA-TGA-ATC-CAG-3′; mNeo-reverse, 5′-TAT-CCA-TCA-TGG-CTG-ATG-CAA-3′. After preheating at 94°C for 2 min, 30 cycles were run, with denaturation at 94°C for 30 s, annealing at 55°C for 1 min, elongation at 72°C for 1min and a final extension at 72°C for 1 min. The Cx43-forward and Cx43-reverse primers yielded a product of 520 bp, and the combination of Neo and Cx43 primers yielded

two products of 520 bp (Cx43) and 294 bp (Neo). PCR products were then loaded on a 1.5% agarose gel containing ethidium bromide at 0.34 μM for electrophoresis. Wild-type newborn mice were identified by the presence of a single band at 520 bp, whereas heterozygous animals were characterized by two bands at 520 and 294 bp, respectively.

All experimental procedures received approval from the local animal care committee and were conducted in accordance with the guidelines of the European Union (CEE 86–609).

OB Cell Isolation and Culture

Once genotyping was performed, calvaria of each genotype were pooled and digested sequentially under gentle agitation at 37°C using a three-step process (0.5% trypsin/1.5% EDTA solution for 5 min, 1 mg/ml collagenase II solution for 5 min followed by three 1 mg/ml collagenase digestions for 15 min). Cells isolated by the last three digestions were combined as an OB population after discarding bone debris by filtration (pore diameter = 37 μm). OB cells were then resuspended in DMEM supplemented with 20% fetal calf serum (FCS), 580 mg/l glutamine, 100 mIU/ml penicillin and 100 μg/ml streptomycin and cultured in 25-cm^2 flasks at 150×10^3 cells/ml at 37°C in a humidified environment with 5% CO_2 and 95% air. After confluence, cells were trypsinized in a 0.1% trypsin/0.3% EDTA solution. Cells were resuspended in DMEM supplemented with 10% FCS, 580 mg/l glutamine, 100 mIU/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml ascorbic acid and 10 mM sodium β-glycerophosphate and cultured at 50×10^3 cells/ml in 35-mm plastic dishes at 37°C in a humidified environment with 5% CO_2 and 95% air. Four hours after seeding, careful washing was done, and thereafter, media were renewed daily. To characterize the effect of partial deletion of Cx43 in $Cx43^{+/-}$ OB cells, Western blot and gap-FRAP analyses were performed after 4 days of culture. The expression level of Cx43 protein was determined following a procedure previously described (Cronier et al., 2003). Briefly, after extraction in phosphate-buffered saline (PBS) containing 0.5% sodium deoxycholate, 20% SDS, 0.1% Triton X-100 and protease inhibitors, total OB proteins (30 μg) were separated on 10% polyacrylamide-SDS gel, electroblotted on nitrocellulose membrane and analyzed by Western blotting with monoclonal antibody against Cx43 (1:1,000; Transduction Laboratory, Lexington, KY). The corresponding antigen was detected after incubation with an alkaline phosphatase-coupled secondary antibody at 1:10,000 (Promega, Madison, WI). Densitometric analyses were performed using VisioL@b2000 software (Biocom, Les Ulis, France). The degree of cell-to-cell diffusion was assessed by the gap-FRAP method, developed by Wade, Trosko & Schindler (1986), using 6-carboxyfluorescein as fluorescent probe on a confocal microscope (FV 1000 Olympus IX-81, Tokyo, Japan). Briefly, tested cells were photobleached by strong laser pulses (405 nm) and digital images of the fluorescent emission were recorded at regular intervals (30 s) for 10 min and stored for subsequent analysis. When the bleached cells were connected to unbleached contiguous cells, fluorescence recovery following a slow exponential time course was measured; the kinetic analysis allows determination of the diffusion rate constant (k), which corresponds to the relative permeability of the gap junctions between the tested cells (Cronier et al., 2003).

Recording of $[Ca^{2+}]_i$ Transients

To analyze the impact of Cx43 partial deletion on calcium homeostasis, variations of free $[Ca^{2+}]_i$ were measured using confocal microscopy (MRC 1024; Bio-Rad, Richmond, CA) after short ET-1 perfusion (100 nM). The Ca^{2+} indicator Fluo-4 was used, for which fluorescence emission at 516 nm after excitation at 488 nm depends on the Ca^{2+} intracellular level. Briefly, after 2 days, cells cultured on 30-mm glass coverslips were loaded 20 min in the dark in a Tyrode solution (in mM: 144 NaCl, 5.4 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 0.3 NaH_2PO_4, 5 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES], 5.6 glucose, pH 7.4) containing the lipophilic form of the dye (Fluo-4/AM ester dissolved in dimethyl sulfoxide 0.1%) at a concentration of 2.7 μM. After carefully washing off the unincorporated fluorogenic dye, variations of $[Ca^{2+}]_i$ with time were measured in defined areas located in the center of OB cells. By means of a home-made gravity-based microperfusion system, ET-1 was rapidly applied using a streamline flow directed onto the OB cells under investigation from the opening of a stainless steel capillary tube (internal diameter 50 μm) positioned in the bath. All experiments were conducted at room temperature (20 ± 1°C) in Tyrode solution.

Fluo-4 signals were not calibrated in terms of absolute values since it was not necessary for the monitoring of variations of $[Ca^{2+}]_i$ levels. However, fluorescence data were normalized to baseline for each cell.

Real-Time RT-PCR

Expression levels of ETA and ETB receptor subtypes were assessed after 4 days of culture. Total RNA was then isolated using RNeasy Plus Mini kit (Qiagen, Valencia City, CA) according to the instructions of the manufacturer. Plastic dishes (35 mm) were harvested with 400 μl lysis buffer, and cell lysis was performed using a 2-s pulse with a probe sonicator. RNA (5 μg) was reverse-transcribed using the RETROscript kit (Ambion, Huntingdon, UK).

The reactions were set up in duplicate in 20 µl total volume with 5 pmol of each primer, 10 µl of 2x SYBRgreen Master Mix and 1 µl of template. The PCR cycle was as follows: 95°C for 10 min, 35 cycles of 95°C for 15 s and 60°C for 1 min, and a melt curve analysis was performed at the end of each experiment to verify that a single product per primer pair was amplified. The amplification and analysis were performed using an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Samples were compared using the relative C_T method. We also determined that a no reverse-transcriptase control reaction yielded no amplification product. Specific primers for ETA receptor, ETB receptor and housekeeping gene (P0) were synthesized by Eurogentec (Liège, Belgium): ET receptor subtype A (XM_134499, mETAR) forward, 5′-ATC-GGG-ATC-CCC-TTG-ATT-AC-3′; mETAR-reverse, 5′-ACA-GCA-ACA-GAG-GCA-GGA-CT-3′; ET receptor subtype B (BC026553, mETBR) forward 5′-TGT-TCG-TGC-TAG-GCA-TCA-TC-3′; mETBR-reverse 5′-AGC-AAT-CTG-CAT-ACC-GCT-CT-3′ (Scarparo et al., 2006); and housekeeping gene encoding ribosomal protein P0 (BC070194) forward 5′-ATG-CCC-AGG-GAA-GAC-AGG-GC-3′ and P0-reverse 5′-CCA-TCA-GCA-CCA-CAG-CCT-TC-3′.

Statistical Analysis

Reported calcium data are expressed as mean ± standard error of the mean (SEM) of fluorescence amplitude between basal level and peak level, with n being the number of OB cells tested. Peak values were compared with Student's t-test for unpaired data, and differences were considered significant at $p \leq 0.05$ (ns, not significant; $*p \leq 0.01$, $**p \leq 0.001$). All statistical tests were performed by means of Origin software version 6.0 (Microcal Software, Northampton, MA).

Results

Cx43 Expression and Function in $Cx43^{+/-}$ and $Cx43^{+/+}$ OB Cells

Under the experimental conditions used here, after 4 days of culture, no evident morphological differences were observed between $Cx43^{+/+}$ and $Cx43^{+/-}$ OB cells (Fig. 1**A,B**). For both genotypes, various OB differentiation states were noted from proliferative OB cells to large differentiated cells. However, as revealed by Western blot analyses, a significant difference was measured in Cx43 expression level, with a 50% decrease in $Cx43^{+/-}$ cells compared to their counterparts (Fig. 1**C,D**). Inter-OB communication was assessed by the gap-FRAP method as previously described (Cronier et al., 2002). Coupled cells were then characterized by an exponential time course of fluorescence recovery from neighboring cells into a photobleached tested cell (Fig. 1**E,F**). After the same period of culture, functional communication in $Cx43^{+/-}$ OB cells presented a 60.3% reduction compared to $Cx43^{+/+}$ OB cells, as quantified by the mean values of diffusion rate constants (Fig. 1**G**). Therefore, partial deletion of the Cx43 gene in mice induced a reduction by half of expression and function of the gap junctional protein in OB cells.

Effect of ET-1 on $[Ca^{2+}]_i$ of $Cx43^{+/-}$ and $Cx43^{+/+}$ OB Cells

ET-1 (100 nM) was effective in inducing a Ca^{2+} response characterized by a rapid increase in fluorescence intensity upon the short perfusion of the peptide in both genotypes (Fig. 2). However, the peak amplitude evoked by ET-1 was significantly lower ($p \leq 0.001$) in $Cx43^{+/-}$ ($n = 470$) compared to $Cx43^{+/+}$ ($n = 538$) cells. The nature of ET-1 receptor subtypes was investigated using a pharmacological approach with specific antagonists and agonist. As shown in Figure 3(**A–D,G**), prior and concomitant perfusion of OB cells with a specific ETA receptor antagonist (BQ123, 1 µM) or ETB receptor antagonist (BQ788, 1 µM) totally abolished the Ca^{2+} transient in the presence of ET-1 for both genotypes ($n = 38$ and 24 for $Cx43^{+/+}$ and $Cx43^{+/-}$, respectively). It should be noted that reversibility within the assay duration was obtained after BQ788 removal, contrary to the BQ123 situation. In both genotypes, peak amplitudes elicited by ET-3 (100 nM, $n = 132$), which has low affinity for ETA subtype, were significantly lower ($p \leq 0.001$) than those in the presence of ET-1 (Fig. 3**E,F,H**). These results supported the dual involvement of ETA and ETB receptors in the short-term effect of ET-1 on OB cells.

To explain the difference due to partial deletion of the Cx43 gene in the ET-1-induced Ca^{2+} response, several assumptions could be postulated. Firstly, to test a possible impact on the expression level of ET receptor subtypes, we performed quantitative RT-PCR using specific primers (Fig. 4). Interestingly, there was no significant difference in expression profiles between the two genotypes ($n = 9$), but the ETA subtype was always expressed at a higher level (20-fold). These data are consistent with those obtained during the ET3 experiment and confirm the predominance of the ETA subtype and the absence of a significant effect of partial deletion of the Cx43 gene on ET receptor expression. Secondly, we postulated that the signal transduction pathway could be affected by the Cx43 deletion and especially the coupling of ET receptor to phospholipase C (PLC). As shown in Figure 5, preincubation (10 min) of OB cells with U73122 (2 µM), a membrane-permeable PLC inhibitor, dramatically decreased

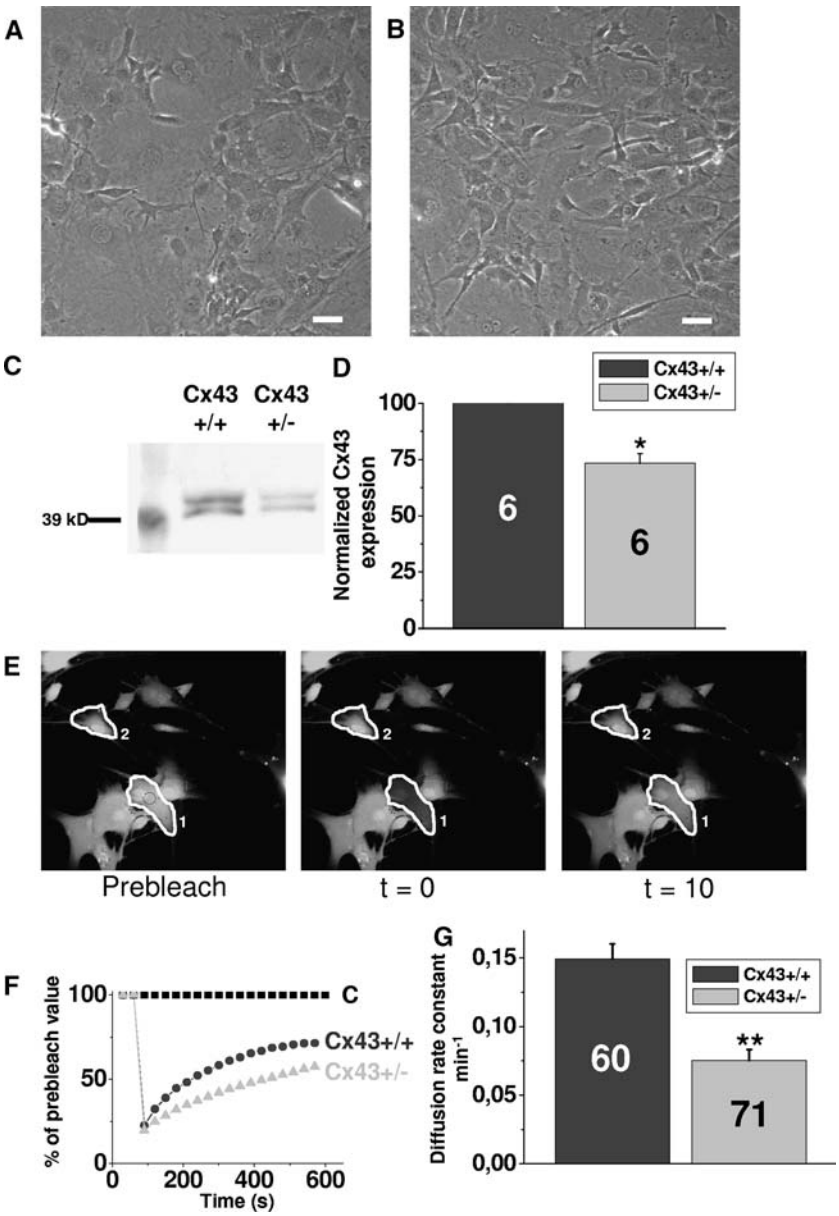

Fig. 1 Cx43 expression and function in cultured calvarial OB cells after 4 days of culture. Phase-contrast micrographs of $Cx43^{+/+}$ (**A**) and $Cx43^{+/-}$ (**B**) OB cells. Scale bars = 50 μm. (**C**) Western blot analysis of Cx43 expression levels in $Cx43^{+/+}$ and $Cx43^{+/-}$ OB cells. Results are representative examples of six separate experiments. (**D**) Normalized densitometric analyses of Western blots showing that Cx43 protein amount is significantly decreased in $Cx43^{+/-}$ OB cells *vs.* control. (e-g) Functional coupling in $Cx43^{+/+}$ and $Cx43^{+/-}$ cells measured by the gap-FRAP method. (**E**) Digital images of fluorescence distribution in OB cells during gap-FRAP experiment: *Prebleach* just after photobleach (t = 0) and after fluorescence redistribution (t = 10), *polygon 1* photobleached tested cell, *polygon 2* unbleached control cell for correction of the loss of fluorescence due to repeated scanning and dye leakage. (**F**) Examples of corrected recovery curves expressed as the percentage of prebleach value *vs.* time in $Cx43^{+/+}$ and $Cx43^{+/-}$ cells and control (*C*) cell. (**G**) Diffusion rate constant (*k*) of $Cx43^{+/+}$ and $Cx43^{+/-}$ OB cells. Numbers of investigated cells are indicated inside the bars. $^{*}p \leq 0.01$, $^{**}p \leq 0.001$

($p \leq 0.001$) the ET-1-induced Ca^{2+} rise ($n = 44$ and 36 for $Cx43^{+/+}$ and $Cx43^{+/-}$ OB cells, respectively), suggesting a lack of alteration at this level due to the reduced connexin expression. Then, we investigated if in $Cx43^{+/-}$ OB cells the release of intracellular calcium was modified. In Ca^{2+}-free medium, ET-1 was able to induce a spike in $[Ca^{2+}]_i$ whatever the genotype (Fig. 6**A,B**). However, in $Cx43^{+/+}$ OB cells, the Ca^{2+} rise was less than that measured in control medium (–28.5%, $p \leq 0.01$), whereas in $Cx43^{+/-}$ OB cells, no significant difference was observed compared to control (Fig. 6**G**). To further estimate the real implication of calcium stores in response to ET-1, thapsigargin (5 μM) was used to specifically block sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA), leading to depletion of Ca^{2+} stores and 2-AminoethoxydiPhenyl Borate(APB) (100 μM) to completely block inositol 1,4,5-trisphosphate (IP_3) receptors. Whatever the genotype, the presence of 2-APB completely abolished the ET-1-stimulated $[Ca^{2+}]_i$ increase (Fig. 6**C,D,H**; $n = 36$). Similarly, in both genotypes, thapsigargin (Fig. 6**E,F**) dramatically reduced ($p \leq 0.001$) the ET-1-induced Ca^{2+} peak ($n = 36$ and 48 in $Cx43^{+/+}$ and $Cx43^{+/-}$ OB cells, respectively), confirming the dual origin of calcium in the peptide response (Fig. 6**I**). However, the rise of $[Ca^{2+}]_i$ elicited in the presence of thapsigargin (5 μM) alone was significantly less pronounced in $Cx43^{+/+}$ OB cells than the one due to ET-1 (100 nM). In $Cx43^{+/-}$ OB cells, there was no significant difference in $[Ca^{2+}]_i$ response between ET-1 and

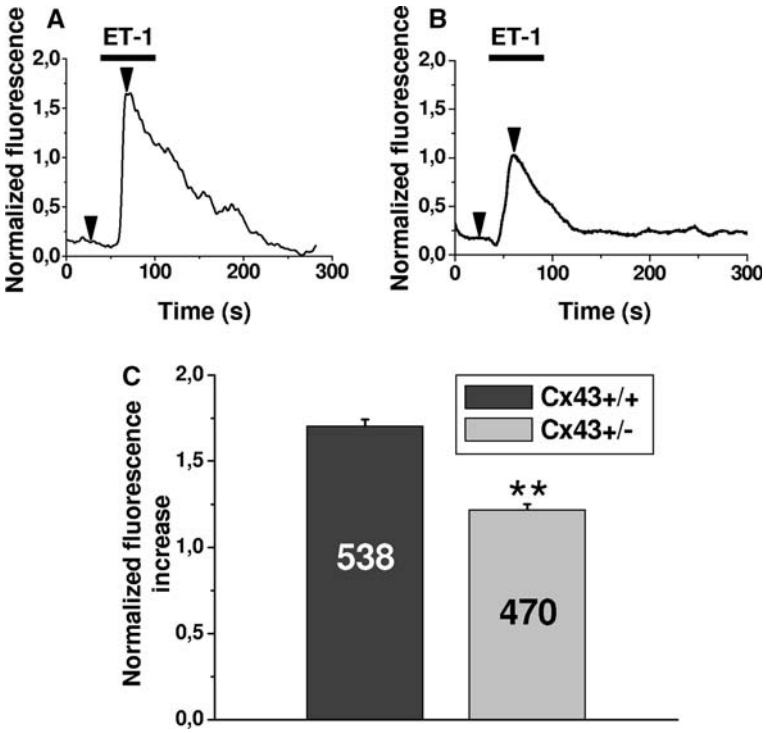

Fig. 2 Difference in ET-1-induced Ca^{2+} response between $Cx43^{+/+}$ and $Cx43^{+/-}$ OB cells after 2 days of culture. Representative recordings of $[Ca^{2+}]_i$ changes in response to short ET-1 (100 nM) perfusion in $Cx43^{+/+}$ (**A**) and $Cx43^{+/-}$ (**B**) OB cells. *Arrowheads* indicate the time of measurements for normalized fluorescence increase expressed in **C**. (**C**) Average peak data obtained in $Cx43^{+/+}$ and $Cx43^{+/-}$ OB cells. Data are mean ± SEM, and numbers of experiments are indicated inside of the bars. $^{**}p \leq 0.001$

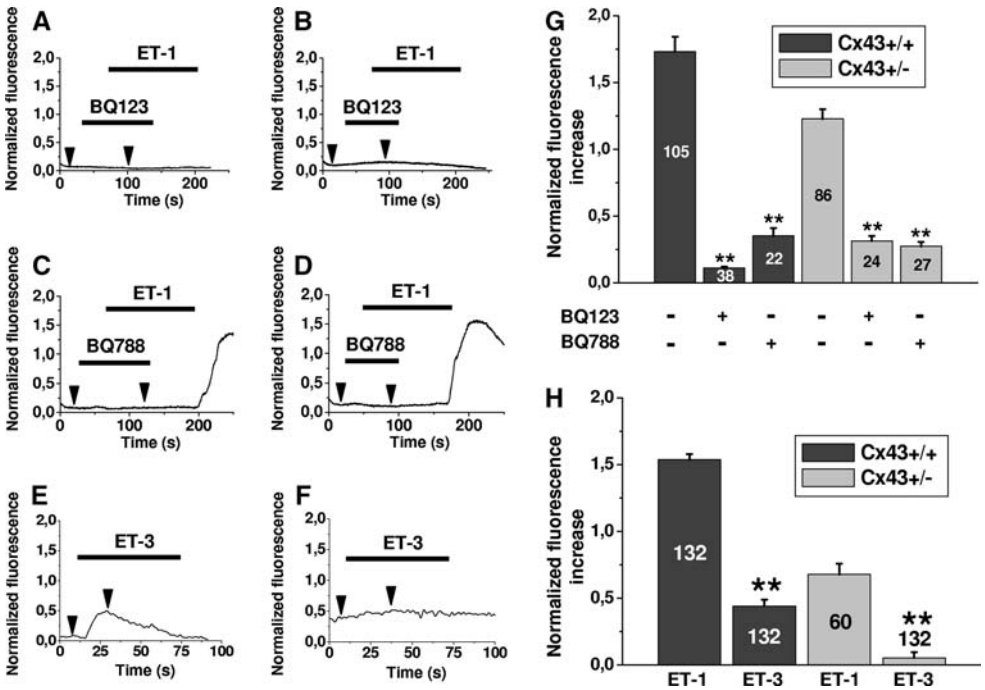

Fig. 3 Pharmacological characterization of ET-1 receptor subtypes in cultured OB cells. (**A–D**) Representative recordings of the effects of BQ123 (1 μM, **A,B**) and BQ788 (1 μM, **C,D**) on the $[Ca^{2+}]_i$ variations elicited by ET-1 (100 nM) in $Cx43^{+/+}$ (**A,C**) and $Cx43^{+/-}$ (**B,D**) OB cells. Representative recordings of $[Ca^{2+}]_i$ changes in response to short ET-3 (100 nM) perfusion in $Cx43^{+/+}$ (**E**) and $Cx43^{+/-}$ (**F**) OB cells. *Arrowheads* indicate the time of measurements for normalized fluorescence increase expressed in (**G,H**). (**G**) Average peak data obtained in $Cx43^{+/+}$ and $Cx43^{+/-}$ OB cells after BQ treatments. (**H**) Average peak data obtained in $Cx43^{+/+}$ and $Cx43^{+/-}$ cells after ET-3 stimulation. Data are mean ± SEM, and numbers of experiments are indicated inside or on top of the bars. $^{**}p \leq 0.001$ *vs.* each genotypic control

thapsigargin, suggesting a low participation of calcium influx in this situation. Finally, in order to clarify which transmembrane channels were involved in the ET-1-induced $[Ca^{2+}]_i$ response, a panel of pharmacological agents was used during the ET-1 perfusion in the vicinity of $Cx43^{+/+}$ or $Cx43^{+/-}$ OB cells. As illustrated in Figure 7, only the dihydropyridine compound nifedipine (L-type Ca^{2+} channel blocker, 1 μM) presented a differential effect according to the level of Cx43 expression (Fig. 7**E,F,I**). Indeed, a 26% reduction was measured in $Cx43^{+/+}$ OB cells ($p \leq 0.01$), whereas no significant effect was demonstrated in $Cx43^{+/-}$ OB cells ($n = 96$ and 83, respectively). Ni^{2+} (1 mM, nonspecific inhibitor of calcium channels; Fig. 7**A,B,G**) and SK&F96365 (30 μM, store-operated calcium channel and nonselective cationic channel blocker; Fig. 7**C,D,H**) had similar effects on both genotypes.

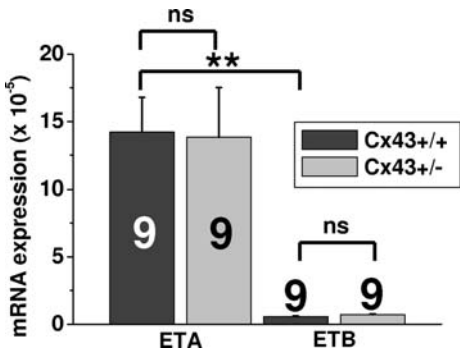

Fig. 4 Partial Cx43 gene deletion does not alter ET receptor subtype expression levels. Real-time RT-PCR revealed that ETA receptor subtype mRNA levels were significantly higher than ETB levels whatever the genotype. However, no significant difference in expression of both receptor subtypes was measured between $Cx43^{+/+}$ and $Cx43^{+/-}$ OB cells ($n = 9$). Data correspond to the mean ± SEM of three independent triplicate experiments. *ns* not significant, $^{**}p \leq 0.001$

Discussion

Data obtained in the present study revealed for the first time that partial Cx43 gene deletion induced a significant decrease of ET-1-induced $[Ca^{2+}]_i$ mobilization. This altered response to ET-1 was not linked to changes in ETA or ETB receptor subtype expression. Indeed, both receptor subtypes are functional in $Cx43^{+/-}$ and $Cx43^{+/+}$ OB plasma membranes even if ETA receptors represented the predominant form, whatever the genotype. Pharmacological investigations led us to demonstrate that the significant difference in $[Ca^{2+}]_i$ peak amplitude during the ET-1 action was associated with decreased calcium influx involving L-VSCCs whereas calcium release from intracellular stores and implication of PLC were not affected by the reduced expression of Cx43.

Among the multiple intracellular signaling pathways coupled to ET receptors (Green et al., 1994; Sokolovsky, 1995), it has been previously shown that ET-1 produced a $[Ca^{2+}]_i$ rise, mainly implicating the ETA receptor subtype compared to ETB, in OB cell lines (Suzuki et al., 1997; Hiruma et al., 1998; Zach, Windischhofer & Leis, 2001) and in rodent calvarial OB cells (Takuwa, Masaki & Yamashita, 1990; Semler et al. 1995; Kitten et al., 1997;

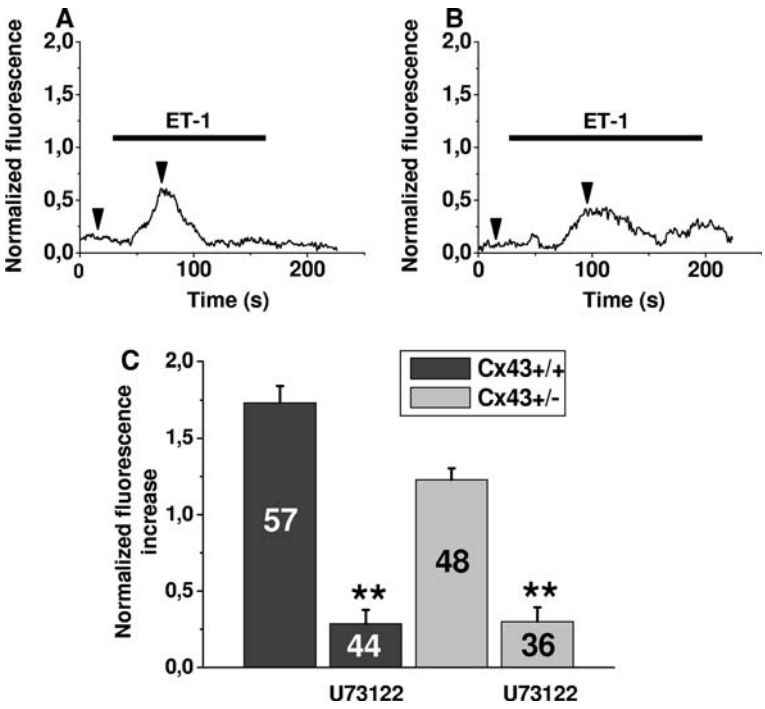

Fig. 5 Coupling of ET-1 receptors to PLC pathway in OB cells is not affected by partial deletion of the Cx43 gene. (**A,B**) Representative recordings of $[Ca^{2+}]_i$ changes evoked by ET-1 (100 nM) after 10-min pretreatment with U73122 (2 μM). *Arrowheads* indicate the time of measurements for normalized fluorescence increase expressed in **C**. (**C**) Average peak data obtained in $Cx43^{+/+}$ and $Cx43^{+/-}$ cells after U73122 preincubation *vs.* control. Data are mean ± SEM, and numbers of experiments are indicated inside of the bars. $^{**}p \leq 0.001$

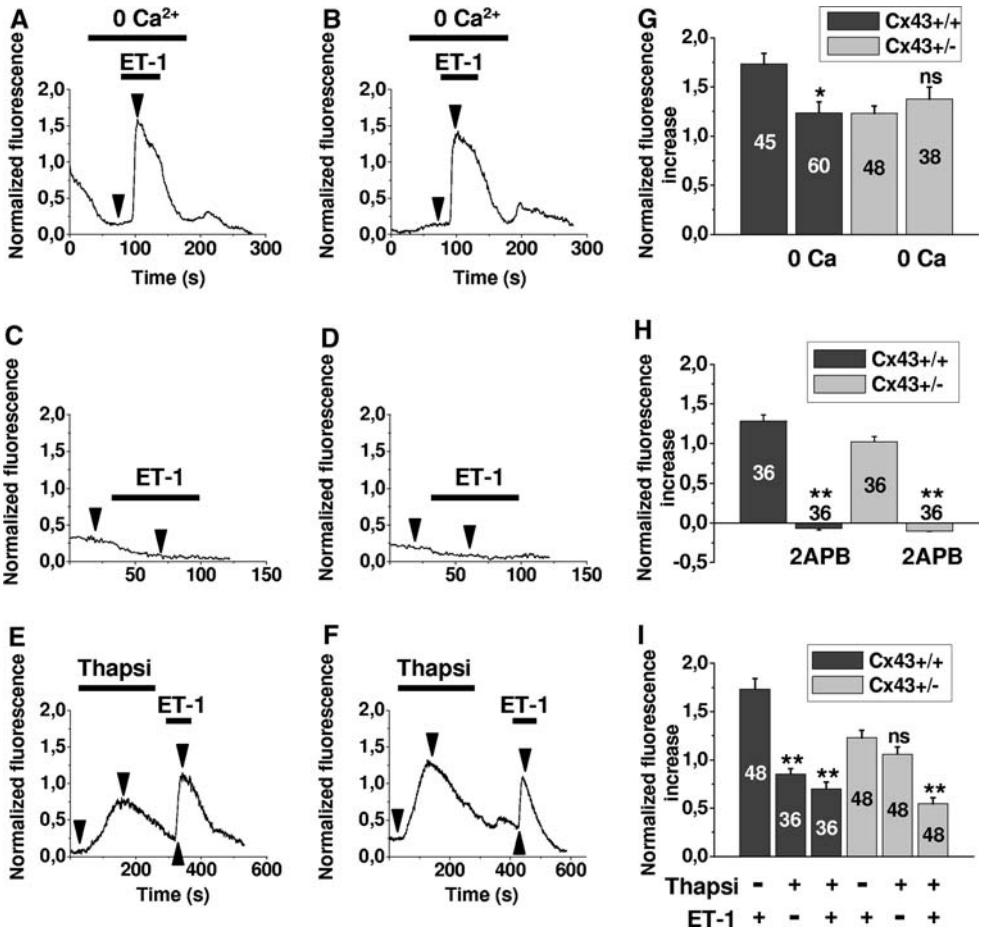

Fig. 6 Decreased Cx43 expression and function in OB cells does not change the implication of intracellular calcium stores in the ET-1 response. Representative recordings of the effects of Ca^{2+}-free medium (**A,B**), 2-APB (100 μM, **C,D**) and thapsigargin (5 μM, **E,F**) on the $[Ca^{2+}]_i$ changes elicited by ET-1 (100 nM) in $Cx43^{+/+}$ (**A,C,E**) and $Cx43^{+/-}$ (**B,D,F**) OB cells. *Arrowheads* indicate the time of measurements for normalized fluorescence increase expressed in **G**, **H** and **I**. Average peak data obtained in $Cx43^{+/+}$ and $Cx43^{+/-}$ cells in the presence of Ca^{2+}-free medium (**G**), after 2-APB treatment (**H**) and after thapsigargin treatment (**I**). Data are mean ± SEM, and numbers of experiments are indicated inside or on top of the bars. *ns* not significant, $*p \leq 0.01$, $**p \leq 0.001$

Hagiwara et al., 1999). In the present study, as described by Semler, Morris & Stern (2000), the real-time RT-PCR technique allowed us to support the presence of ETB subtype in cultured calvarial cells, and its implication in ET-1-induced calcium response was confirmed by means of specific antagonists and agonist. In most of the cell types, ET-1 commonly induced a $[Ca^{2+}]_i$ elevation caused by PLC activation and IP_3 generation, leading to release of sequestrated calcium ions and to Ca^{2+} influx through plasma membranes by voltage-sensitive and -insensitive calcium or cationic channels (Berridge et al., 2000; Kawanabe et al., 2001; Niger, Malassiné & Cronier, 2004). However, no evidence of ET-1 activation of L-VSCCs has been observed (Zach et al., 2001) by means of fluorimetry in isolated MC3T3-E1 cells in suspension (pre-OB cell line). It must be pointed out that numerous biochemical and molecular studies have demonstrated the presence of voltage-operated (L- and T-types) calcium channels in OB cells (Caffrey & Farach-Carson, 1989; Li et al., 2003; Shao, Alicknavitch & Farach-Carson, 2005; Bergh et al., 2006). In our experimental conditions, nifedipine, a specific L-VSCC blocker, had no significant effect on $Cx43^{+/-}$ OB cells, whereas it was effective in $Cx43^{+/+}$ OB cells. This result suggests that L-VSCCs are involved in the ET-1-induced Ca^{2+} rise observed in the absence of Cx43 deletion.

Endothelin excepted, other biologically active substances present in the bone microenvironment (PTH, PTH-related protein, vitamin D_3, estradiol) were demonstrated to modulate calcium influx through L-VSCCs in OB cells (Lieberherr et al., 1993; Lalonde et al., 2001; Li et al., 2003; Bergh et al., 2006). For example, *in vitro* studies have shown that PTH enhanced $[Ca^{2+}]_i$ in OB cells subjected to fluid shear stress and that this stimulatory effect was attenuated by L-type calcium channel blockers (Ryder & Duncan, 2001). Voltage-independent cationic channels were also demonstrated in OB cells, especially store-operated and capacitative calcium entry (Zach et al., 2001; Baldi, Vazquez & Boland, 2002; Santillan et al., 2004); but no data were available on the ET-1 effect on these channels. Nevertheless, in the present study, the blocking effect of SK&F96365 during ET action suggested the participation of these channels in the Ca^{2+} response for both genotypes.

As postulated in recent studies describing pannexin transfection (connexin homologues) in human cell lines

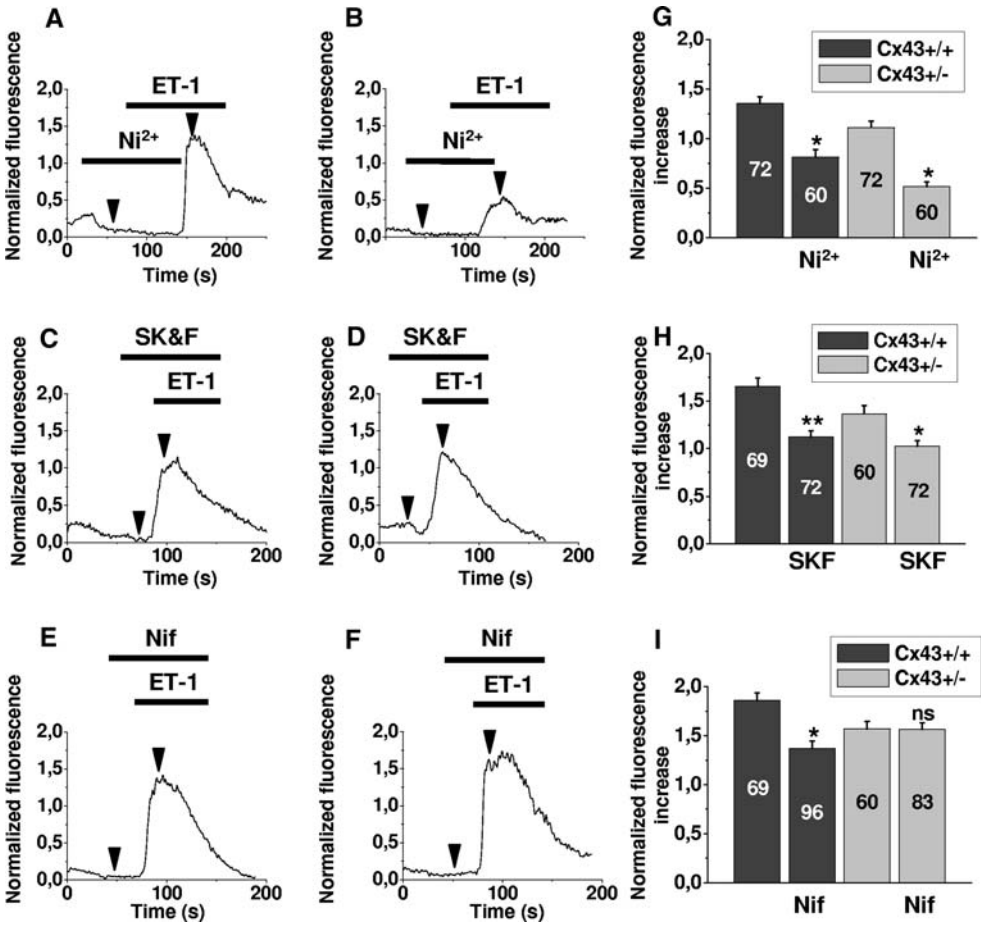

Fig. 7 Difference between $Cx43^{+/+}$ and $Cx43^{+/-}$ OB cells in transmembrane channels involved in ET-1-induced Ca^{2+} response. Representative recordings of the effects of Ni^{2+} (1 mM, **A,B**), SK&F96365 (30 μM, **C,D**) and nifedipine (1 μM, **E,F**) on the $[Ca^{2+}]_i$ changes elicited by ET-1 (100 nM) in $Cx43^{+/+}$ (**A,C,E**) and $Cx43^{+/-}$ (**B,D,F**) OB cells. *Arrowheads* indicate the time of measurements for normalized fluorescence increase expressed in **G**, **H** and **I**. Average peak data obtained in $Cx43^{+/+}$ and $Cx43^{+/-}$ cells in the presence of Ni^{2+} (**G**), SK&F96365 (**H**) and nifedipine (**I**). Data are mean ± SEM, and numbers of experiments are indicated inside of the bars. *ns* not significant, $*p \leq 0.01$, $**p \leq 0.001$

(Vanden Abeele et al., 2006), this type of transmembrane protein could form hemichannels which seem to be present in the endoplasmic reticulum (ER) membrane and to contribute to the ER-calcium leak. The question is whether Cx43 could form such hemichannels in OB ER membranes responsible for the observed phenomenon. In our experimental conditions, decreased expression of Cx43 had no effect on calcium mobilization from intracellular stores after ET-1 and thapsigargin treatments compared to wild-type OB cells, suggesting that Cx hemichannels were not implicated in the ER Ca^{2+} release. The presence and implication of Cx hemichannels in the control of calcium homeostasis require further investigations. As the lack of Cx43 in OB cells hindered the bone anabolic effect of PTH, the link between Cx43 expression level and OB control by calcium mobilizing factors like ET-1 appears to be important. Thus, Chung et al. (2006) demonstrated that subcutaneous injection of PTH in conditionally OB-deleted Col/Cre;$Cx43^{-/fl}$ mice induced an attenuated anabolic response compared to their wild-type littermates as assessed by bone mineral density and histomorphometric analyses. Finally, as previously demonstrated (Lecanda et al., 2000), the reduced Cx43 expression level could also influence the OB differentiation process, which presents dynamic variations in expression of some partners implicated in calcium homeostasis. Indeed, Lampasso, Chen & Marzec (2006) demonstrated a temporal pattern of expression of protein kinase C isoforms in differentiating MC3T3-E1 cells by immunofluorescence and Western blotting. Moreover, biochemical and pharmacological studies have revealed that $1,25(OH)_2D_3$ treatment of MC3T3-E1 cells leads to a shift in VSCC expression from predominantly L-type in early differentiating OB cells to T-type in terminally differentiated OB cells (Bergh et al., 2006).

In conclusion, our data demonstrate that the Cx43 level of expression and/or function is able to modulate $[Ca^{2+}]_i$ mobilization in a murine OB model. $Cx43^{+/-}$ OB cells, which express a significantly lower level of Cx43, exhibit a reduced Ca^{2+} spike when treated with ET-1. The implications of L-VSCC together with voltage-independent channels were also shown during the ET-1-induced calcium rise by use of pharmacological agents. Even if our data confirm the role of gap junctional intercellular communication in the control of Ca^{2+} homeostasis, further studies will be needed to investigate the real involvement of Cx43 in this phenomenon and particularly its molecular interactions and/or interactive pathways with L-VSCCs.

References

Aubin JE, Liu F, Malaval L, Gupta A (1995) Osteoblast and chondroblast differentiation. Bone 17:77S–83S

Baldi C, Vazquez G, Boland R (2002) Characterization of a 1,25(OH)$_2$-vitamin D$_3$-responsive capacitative Ca^{2+} entry pathway in rat osteoblast-like cells. J Cell Biochem 86:678–687

Baylink DJ, Finkelman RD, Mohan S (1993) Growth factors to stimulate bone formation. J Bone Miner Res 8:S565–S572

Bergh JJ, Shao Y, Puente E, Duncan RL, Farach-Carson MC (2006) Osteoblast Ca^{2+} permeability and voltage-sensitive Ca^{2+} channel expression is temporally regulated by 1,25-dihydroxyvitamin D$_3$. Am J Physiol 290:C822–C831

Berridge MJ, Lipp P, Bootman MD (2000) The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 1:11–21

Caffrey JM, Farach-Carson MC (1989) Vitamin D$_3$ metabolites modulate dihydropyridine-sensitive calcium currents in clonal rat osteosarcoma cells. J Biol Chem 264:20265–20274

Chung DJ, Castro CH, Watkins M, Stains JP, Chung MY, Szejnfeld VL, Willecke K, Theis M, Civitelli R (2006) Low peak bone mass and attenuated anabolic response to parathyroid hormone in mice with an osteoblast-specific deletion of connexin43. J Cell Sci 119:4187–4198

Civitelli R, Ziambaras K, Warlow PM, Lecanda F, Nelson T, Harley J, Atal N, Beyer EC, Steinberg TH (1998) Regulation of connexin43 expression and function by prostaglandin E$_2$ (PGE$_2$) and parathyroid hormone (PTH) in osteoblastic cells. J Cell Biochem 68:8–21

Clapham DE (1995) Calcium signalling. Cell 80:259–268

Cronier L, Defamie N, Dupays L, Theveniau-Ruissy M, Goffin F, Pointis G, Malassiné A (2002) Connexin expression and gap junctional intercellular communication in human first trimester trophoblast. Mol Hum Reprod 8:1005–1013

Cronier L, Frendo JL, Defamie N, Pidoux G, Bertin G, Guibourdenche J, Pointis G, Malassiné A (2003) Requirement of gap junctional intercellular communication for human villous trophoblast differentiation. Biol Reprod 69:1472–1480

Duncan RL, Akanbi KA, Farach-Carson MC (1998) Calcium signals and calcium channels in osteoblastic cells. Semin Nephrol 18:178–190

Green J, Foellmer O, Kleeman CR, Basic MM (1994) Endothelin receptor in osteoblastic cells is coupled to multiple messenger signals. Am J Physiol 267:C1329-C1337

Hagiwara H, Naruse M, Adachi C, Inoue A, Hiruma Y, Otsuka E, Naruse K, Demura H, Hirose S (1999) Ipriflavone down-regulates endothelin receptor levels during differentiation of rat calvarial osteoblast-like cells. J Biochem 126:168–173

Hiruma Y, Inoue A, Shiohama A, Otsuka E, Hirose S, Yamaguchi A, Hagiwara H (1998) Endothelins inhibit the mineralization of osteoblastic MC3T3-E1 cells through the A-type endothelin receptor. Am J Physiol 275:R1099-R1105

Kawanabe Y, Okamoto Y, Enoki T, Hashimoto N, Masaki T (2001) Ca^{2+} channels activated by endothelin-1 in CHO cells expressing endothelin-A or endothelin-B receptors. Am J Physiol 281:C1676–C1685

Kitten AM, Harvey SA, Criscimagna N, Asher M, Lee JC, Olson MS (1997) Osteogenic protein-1 downregulates endothelin A receptors in primary rat osteoblasts. Am J Physiol 272:E967–E975

Lalonde M, Li B, Kline L, Pang P, Karpinski E (2001) Modulation of L-type Ca^{2+} channels in UMR 106 cells by parathyroid hormone-related protein. Life Sci 70:503–515

Lampasso JD, Chen W, Marzec N (2006) The expression profile of PKC isoforms during MC3T3–E1 differentiation. Int J Mol Med 17:1125–1131

Lecanda F, Towler DA, Ziambaras K, Cheng SL, Koval M, Steinberg TH, Civitelli R (1998) Gap junctional communication modulates gene expression in osteoblastic cells. Mol Biol Cell 9:2249–2258

Lecanda F, Warlow PM, Sheikh S, Furlan F, Steinberg TH, Civitelli R (2000) Connexin43 deficiency causes delayed ossification, craniofacial abnormalities, and osteoblast dysfunction. J Cell Biol 151:931–944

Li J, Duncan RL, Burr DB, Gattone VH, Turner CH (2003) Parathyroid hormone enhances mechanically induced bone formation, possibly involving L-type voltage-sensitive calcium channels. Endocrinology 144:1226–1233

Li Z, Zhou Z, Saunders MM, Donahue HJ (2006) Modulation of connexin43 alters expression of osteoblastic differentiation markers. Am J Physiol 290:C1248–C1255

Lieberherr M (1987) Effects of vitamin D$_3$ metabolites on cytosolic free calcium in confluent mouse osteoblasts. J Biol Chem 262:13168–13173

Lieberherr M, Grosse B, Kachkache M, Balsan S (1993) Cell signaling and estrogens in female rat osteoblasts: a possible involvement of unconventional nonnuclear receptors. J Bone Miner Res 8:1365–1376

Niger C, Malassiné A, Cronier L (2004) Calcium channels activated by endothelin-1 in human trophoblast. J Physiol 561:449–458

Niger C, Geneau G, Fiorini C, Defamie N, Pointis G, Mesnil M, Cronier L (2007) Endothelin-1 inhibits human osteoblastic cell differentiation: influence of connexin-43 expression level. J Cell Biochem (in press)

Provot S, Schipani E (2005) Molecular mechanisms of endochondral bone development. Biochem Biophys Res Commun 328:658–665

Reaume AG, de Sousa PA, Kulkarni S, Langille BL, Zhu D, Davies TC, Juneja SC, Kidder GM, Rossant J (1995) Cardiac malformation in neonatal mice lacking connexin43. Science 267:1831–1834

Ryder KD, Duncan RL (2001) Parathyroid hormone enhances fluid shear-induced $[Ca^{2+}]_i$ signaling in osteoblastic cells through activation of mechanosensitive and voltage-sensitive Ca^{2+} channels. J Bone Miner Res 16:240–248

Santillan G, Baldi C, Katz S, Vazquez G, Boland R (2004) Evidence that TRPC3 is a molecular component of the 1α,25(OH)$_2$D$_3$-activated capacitative calcium entry (CCE) in muscle and osteoblast cells. J Steroid Biochem Mol Biol 89–90:291–295

Scarparo AC, Isoldi MC, de Lima LH, Visconti MA, Castrucci AM (2006) Expression of endothelin receptors in frog, chicken, mouse and human pigment cells. Comp Biochem Physiol A Integr Physiol (in press)

Schiller PC, D'Ippolito G, Balkan W, Roos BA, Howard GA (2001) Gap-junctional communication is required for the maturation process of osteoblastic cells in culture. Bone 28:362–369

Semler DE, Morris DL, Stern PH (2000) Endothelin-stimulated Ca^{2+}signaling and endothelin receptor expression are decreased by parathyroid hormone treatment in UMR-106 osteoblastic osteosarcoma cells. Cell Calcium 28:55–64

Semler DE, Ohlstein EH, Nambi P, Slater C, Stern PH (1995) Endothelin-1-evoked calcium transients in UMR-106 osteoblastic osteosarcoma cells are mediated through endothelin-A and endothelin-B receptors. J Pharmacol Exp Ther 272:1052–1058

Shao Y, Alicknavitch M, Farach-Carson MC (2005) Expression of voltage sensitive calcium channel (VSCC) L-type Cav1.2 (alpha1C) and T-type Cav3.2 (alpha1H) subunits during mouse bone development. Dev Dyn 234:54–62

Sokolovsky M (1995) Endothelin receptor heterogeneity, G-proteins, and signaling via cAMP and cGMP cascades. Cell Mol Neurobiol 15:561–571

Stern PH, Tatrai A, Semler DE, Lee SK, Lakatos P, Strieleman PJ, Tarjan G, Sanders JL (1995) Endothelin receptors, second messengers, and actions in bone. J Nutr 125:2028S–2032S

Suzuki A, Shinoda J, Watanabe-Tomita Y, Ozaki N, Oiso Y, Kosawa K (1997) ETA receptor mediates the signalling of endothelin-1 in osteoblast-like cells. Bone 21:143–146

Takuwa Y, Masaki T, Yamashita K (1990) The effects of the endothelin family peptides on cultured osteoblastic cells from rat calvariae. Biochem Biophys Res Commun 170:998–1005

Takuwa Y, Ohue Y, Takuwa N, Yamashita K (1989) Endothelin-1 activates phospholipase C and mobilizes Ca^{2+} from extra- and intracellular pools in osteoblastic cells. Am J Physiol 257:E797–E803

Upham BL, Suzuki J, Chen G, Wang Y, McCabe LR, Chang CC, Krutovskikh VA, Yamasaki H, Trosko JE (2003) Reduced gap junctional intercellular communication and altered biological effects in mouse osteoblast and rat liver oval cell lines transfected with dominant-negative connexin 43. Mol Carcinog 37:192–201

Vanden Abeele F, Bidaux G, Gordienko D, Beck B, Panchin YV, Baranova AV, Ivanov DV, Skryma R, Prevarskaya N (2006) Functional implications of calcium permeability of the channel formed by pannexin 1. J Cell Biol 14:535–546

Wade MH, Trosko JE, Schindler M (1986) A fluorescence photobleaching assay of gap junction-mediated communication between human cells. Science 232:525–528

Yamaguchi DT, Green J, Kleeman CR, Muallem S (1989) Properties of the depolarization-activated calcium and barium entry in osteoblast-like cells. J Biol Chem 264:197–204

Yang X, Karsenty G (2002) Transcription factors in bone: developmental and pathological aspects. Trends Mol Med 8:340–345

Zach D, Windischhofer W, Leis HJ (2001) Endothelin- and sarafotoxin-induced receptor-mediated calcium mobilization in a clonal murine osteoblast-like cell line, MC3T3-E1/B. Bone 28:595–602