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Mechanosensitivity of human osteosarcoma cells and phospholipase C β2 expression

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Abstract

Bone adapts to mechanical load by osteosynthesis, suggesting that osteoblasts might respond to mechanical stimuli. We therefore investigated cell proliferation and phospholipase C (PLC) expression in osteoblasts. One Hertz uniaxial stretching at 4000 μstrains significantly increased the proliferation rates of human osteoblast-like osteosarcoma cell line MG-63 and primary human osteoblasts. However, U-2/OS, SaOS-2, OST, and MNNG/HOS cells showed no significant changes in proliferation rate. We investigated the expression pattern of different isoforms of PLC in these cell lines. We were able to detect PLC β1, β3, γ1, γ2, and δ1 in all cells, but PLC β2 was only detectable in the mechanosensitive cells. We therefore investigated the possible role of PLC β2 in mechanotransduction. Inducible antisense expression for 24 h inhibited the translation of PLC β1 in U-2/OS cells by 35% and PLC β2 in MG-63 by 29%. Fluid shear flow experiments with MG-63 lacking PLC β2 revealed a significantly higher level of cells losing attachment to coverslips and a significantly lower number of cells increasing intracellular free calcium.

Keywords: Phospholipase C β2; Mechanical loading; Bone; Fluid flow; Osteosarcoma; Ecdysone; Antisense

The regulation of reception of mechanical loading in osteoblasts leads to a downstream activation of phospholipase C (PLC) by a less known biophysical/biochemical transducer(s) within 120 s [1]. The activation of PLC might be due to the release of an autocrine cytokine such as IL-4 [2].

Once activated the dynamics of the intracellular free calcium release from intracellular stores are the same as seen upon hormonal PLC stimulation, using, for instance, parathyroid hormone [3]. This is consistent with pharmacological evidence that modulators of the PLC associated GTPase can alter mechanotransduction [4] and indicates that the isoenzyme of PLC in question is of the β-type. Osteoblasts and pre-osteoblasts seem to be the major cellular candidates that regulate and/or undertake bone remodeling after mechanical loading. Downstream regulation of physiological amplitudes and modes (uniaxial) of strain and fluid shear flow seems to be mainly transduced in osteoblasts and other cells by the PLC-PKC pathway [5]. The biophysical nature and site of the mechano-transducer itself is less known. The phosphoinositide-specific phospholipase C (PLC) plays a crucial role in initiating mechano-mediated and receptor-mediated signal transduction by generating two second messenger molecules, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), from phosphatidylinositol-4,5-bisphosphate (PIP_2). Diacylglycerol is responsible for activating a large family of protein kinase C isoenzymes, which catalyze protein phosphorylation. On the other hand, IP_3 binds to a receptor and releases Ca^{2+} from the endoplasmatic reticulum [6,7]. Many other downstream events including prostaglandin

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synthesis, nitric oxide synthesis, and their downstream molecular pathways, such as protein kinase A, protein kinase G, and extracellular receptor kinases (ERKs) can be linked to either PKC activation, PLC activation or both [8–13]. Peake et al. [14,15] showed c-fos and AP-1 upregulation in MG-63 cells and in primary human osteoblasts after mechanical stimulation.

So far, 11 different mammalian PLC isoforms have been described. This family is divided into four subtypes β, γ, δ, and ε, each of which contains different isoenzymes: PLC β1–β4, γ1 and γ2, δ1–δ4, and ε have been identified [10,16]. The isoforms can be divided on the basis of amino acid sequence and their way of activation. G-proteins activate PLC β whereas tyrosine phosphorylation activates the PLC γ-subtype. The regulatory mechanisms of PLC δ are still unknown [9]. For the activation of PLC ε the G-protein $G_{\alpha 12}$ is necessary [10]. The activities of all isoforms of PLC are modified by increases in intracellular free Ca^{2+} ion concentration in vivo. The aim of this study was to identify the isoform of PLC involved in the mechanotransduction in osteosarcoma cells and primary osteoblasts. First, we investigated the mechanosensitivity of the osteosarcoma cell lines MG-63 [17], MMNG/HOS [18], OST [19], U-2/OS [20], and SaOS-2 [21], and primary human osteoblasts for mechanical loading using a 4-point-bending machine [1] and the expression pattern of different isoforms of PLC in these cells. To investigate the reaction of human osteosarcoma cells MG-63 (mechanosensitive) and U-2/OS (not mechanosensitive) to mechanical stimulation, we created a new inducible antisense-expressing system for mechanical loading by expressing specific antisense sequences against PLC β1 and β2. There are only very few studies about the mechanosensitivity of osteosarcoma cells by mechanical loading [4,14,15,22] and none relating the expression of different isoforms of phospholipase C in the cells in this regard in the literature.

In order to examine the feasibility of using the ecdysone system for inducible knockdown of the PLC subtypes β1 and β2, we isolated cDNA fragments of the above-mentioned isoforms and cloned them in a reverse direction into the pIND(SP1)-vector and have attempted to establish steroid hormone inducible isoform-specific PLC suppression using the MG-63 and U-2/OS osteosarcoma cell lines. These cell lines were transfected with the regulatory vector pVgRXR that encodes both the RXR and EcR receptors. Following this first procedure, the cells were further modified by incorporating the pIND(SP1) vector containing the antisense gene of interest, which is under the control of a minimal heat shock promoter, the SP1 promotor, which is activated by the endogenous SP1-transcription factor which is expressed in nearly all mammalian cells (Ecdysone manual of Invitrogen, Netherlands) and five upstream ecdysone-response elements. We report about the proliferation rate of human osteoblasts and sarcoma cell lines after mechanical loading and the participation of PLC β2 in this pathway.

Materials and methods

Cell culture. The human osteoblast-like osteosarcoma cell lines MG-63, HOS/MNNG, OST, U-2/OS, and SaOS-2 were obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA. All the cells were cultured in RPMI 1640 medium (Life Technologies), supplemented with 2 mM glutamine (Life Technologies) and 10% fetal calf serum (FCS; Seromed, Germany). To select transfected cells, the antibiotics G-418 (Life Technologies) and Zeocin (Invitrogen) were employed. Primary human osteoblasts (PHO) and bovine osteoblasts (PBO) were cultured and characterized as previously described by Jones et al. [3].

MTT-cell quantification assay. Human osteoblast-like osteosarcoma cells MG-63, MNNG/HOS, OST, U-2/OS, and SaOS-2, and primary human osteoblasts were seeded at a density of 60,000 cells per cm^2 onto sterile polycarbonate plates, each of which has nine wells of 5 mm thick pure silicone rubber (Dow Corning, Germany) with an area of 0.5 cm^2 on the surface. Cells were grown for 24 h, reaching a confluence of around 60%. On day two, three, and four, one set of plates was bent in a 4-point-bending machine [3] for 30 cycles at 1 Hz and 4000 μstrain (με). Control groups did not receive mechanical stimulation, but were otherwise subjected to the same conditions. After another 24 h of incubation the cell activity was measured by the colorimetric microassay by Mosmann [23]. Ten microliters of tetrazolium salt (MTT: 3-[4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, 5 mg/ml; Sigma, Germany) was added to each well and then incubated for 4 h. The plates were read with a multiwell scanning Titertek Elisa reader (wavelength 540 nm, reference length 630 nm). The relative absorbance was shown as a percent of the corresponding control. The relative absorbance was calibrated against control cell cultures plated at different densities, which were then counted in a Coulter ZM cell counter equipped with Channelyser to control for dead cells. The accuracy of the MTT method was shown to be ±200 cells as previously described [23].

Cloning of the antisense vector against phospholipase C β1 and β2. To obtain an antisense-expression vector against phospholipase C β2 a fragment of the human PLC β2 sequence published by Park et al. [24] was used. A unique DNA fragment at position 160–309 was amplified by PCR as this contains the translation start and has only <10% sequence homology with other members of the PLC β family. For amplification, a set of synthetic oligonucleotide primers were generated: forward primer 5′-CGGAATTCCGACCATGTCTCTGCTCA ACC-3′, reverse primer 5′-CGGGGTACCCAGTATAAGTAGTA GCCCTTA-3′. The primer pair included two restriction sites, *Eco*R1 at the 5′-end of forward primer, *Kpn*I at the 5′-end of reverse primer. The double-stranded cDNA was synthesized directly from the human phospholipase C β2 sequence. PCR conditions were: 94 °C/5 min; 30 cycles of (94 °C/30 s; 55 °C/60 s; 72 °C/60 s). The amplified cDNA was purified and cloned into the ecdysone-expression vector pIND(SP1). Both the PLC fragment and the pIND(SP1) vector were cut with *Kpn*I and *Eco*R1, and the PLC fragment was ligated into the vector. Under the same conditions and methods, the antisense-expression vector against PLC β1 was cloned using the fragment 365–514 of PLC β1 cDNA [25] with the following PCR primers: forward 5′-CGGAATTC CGCACCATGTCTCTGCTCAACC-3′; reverse 5′-CGGGGTACCC AGTATAAGTAGTAGCCCTTA-3′.

Sequence analysis. For sequence analysis the T7 DNA-Sequenase-System from USB, Germany, was used. Ten different clones were analyzed with the ecdysone forward primer (Invitrogen):

5′-CTCTGAATACTTTCAACAAGTTAC-3′ according to the manufacturer's protocol. All clones contained the pIND(SP1) vector that included the PLC fragment in the antisense position.

Transfection. Suspensions of 10^5 MG-63 or U-2/OS cells were cultured on glass coverslips for 24 h in standard medium. Cells were transfected with the liposome-mediated transfection kit from Gibco-BRL containing the lipofectamin reagent. One microgram of the cloned plasmid vector pIND(SP1) with antisense insert against phospholipase C β1, respectively, β2 and vector pVgRXR were mixed with lipofectamin in serum-free medium and incubated for 30 min at room temperature according to manufacturer's instructions. Cells were rinsed with serum free medium followed by incubation with the transfection mix for 4 h at 37 ° C. Subsequently, the mix was replaced with standard medium. The cells were grown for 24 h before adding the antibiotics. Several clones were isolated based on their resistance to Zeocin and G-418. Antisense expression was analyzed first with RT-PCR and later by Western blotting.

RT-PCR. Total RNA was extracted as described by Chomczynski and Sacchi [26]. Two micrograms of total RNA from MG-63 and U-2/OS cells, and cells transfected with pVgRXR and pIND(SP1) vector from the cell lines was converted to cDNA employing Superscript II reverse transcriptase (Life Technologies), according to manufacturer's protocol. RT-Primer was 5′-CGGGGTACCCAGTATAAGTAGT AGCCCTTA-3′ to reverse transcribe only antisense-RNA against phospholipase C β2 in a final volume of a 20 μl-reaction. Following first-strand cDNA synthesis, the RT-reaction was stopped by warming up to 99 °C for 5 min. 0.5 μl of this reaction was used for PCR with the primers: forward 5′-CGGAATTCCGCACCATGTCTCTGCTCA ACC-3′; reverse 5′-CGGGGTACCCA-GTATAAGTAGTAGCCCT TA-3′. The conditions of the PCR were: 94 °C/5 min; 30 cycles of (94 °C/30 s; 55 °C/60 s; 72 °C/60 s). The reaction was carried out using 1 U *Taq*-polymerase (Perkin-Elmer, USA) in 50 μl using 5 μl of 10× buffer, containing Tris/HCl, pH 8.4, 100 mM; KCl, 500 mM; 3 μl $MgCl_2$, 25 mM, a final concentration of 300 μM dNTPs and 100 ng of each primer. The PCR products were visualized by electrophoresis on a 1% agarose gel in 1× TBE-buffer (Tris 1 M, boric acid 1 M, and EDTA 20 mM, pH 8.3). For RT-PCR of phospholipase C β1, the RT-primer was 5′-CGGGGTACCCTCCTCAAAATAATTGGAGTA-3′ and the PCR primers were: forward primer 5′-CGGAATTCCAGCCCAGAT GAGCCCAGATGG-3′ and reverse primer 5′-CGGG-GTACCCTC CTCAAAATAATTGGAGTA-3′ under the same conditions as for phospholipase C β2.

Western blotting. The MG-63 and U-2/OS cells, stably transfected with the pVgRXR and the antisense-expression vectors, were cultured with ponasterone A for different periods of time. Then the cells were lifted from the glass sheets and washed twice with ice-cold PBS. The cells were homogenized by ultrasonic treatment for 3 × 20 s and centrifuged for 10 min at 3.000 rpm. Then the pellet was homogenized in a lysis buffer containing Tris/HCl (pH 7.5) 20 mM, KCl 100 mM, EGTA 0.5 mM, EDTA 1 mM, Na-vanadate 1 mM, Na-molybdate 1 mM, calyculine A 1 μM, DTT 1 mM, and Triton X-100 0.3% for 1 h on ice. The lysate was cleaned from debris by centrifugation at 100,000*g* for 30 min. Protein concentration of the supernatants was measured with the BCA reagent (Pierce Chemical, USA). For the detection of PLC expression in wild-type MG-63, MNNG/HOS, OST, U-2/OS, and SaOS-2, the same conditions were used.

For the detection of phospholipase C β1, 50 μg of soluble lysate protein was separated on a 6% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was probed with rabbit polyclonal antibody against phospholipase C β1 in a final concentration of 0.5 μg/ml (Santa Cruz, USA). Concentrations for rabbit polyclonal PLC β2, β3, δ1, and δ2 antibodies were 1.0 μg/ml, for rabbit polyclonal PLC β4, γ1, and γ2 antibodies 0.5 μg/ml (Santa Cruz, USA), probed under the above-mentioned conditions. Phospholipase C isoenzymes were visualized with the use of an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Sigma, Germany) and the NBT/BCIP-system.

Northern blotting. Total RNA was extracted as described by Chomczynski and Sacchi [26]. Twenty micrograms of total RNA from the human osteoblast-like osteosarcoma cell lines MG-63, HOS/MNNG, OST, U-2/OS, and SaOS-2 was electrophoresed on a 1.3% agarose gel containing 2.2 M formaldehyde and stained with ethidium bromide for visualization of ribosomal RNA (rRNA [28S and 18S]). Bands of rRNA were checked to confirm equal loading of RNA in the gel. The RNA was transferred and fixed onto Hybond-N nylon membrane (Amersham, UK) according to the manufacturer's recommendations. cDNA fragments of phospholipase C isoenzymes were amplified by PCR using primer pairs from the start codon region of the isoform with a total product length of around 1000 bp (for conditions of PCR see above "RT-PCR"). Fragments were purified on agarose gel and labeled with [α-^{32}P]desoxycytosine trisphosphate and [α-^{32}P]desoxythymidine trisphosphate (each 3000 Ci/mmol; Amersham, UK) by random hexanucleotide-primed second strand synthesis (Fermentas, UK) according to manufacturer's recommendations. The blots were hybridized at 65 °C in Quickhyb hybridization buffer (Stratagene, UK) and washed under stringent conditions. For the last wash stringent conditions were used (0.1× SSC, 0.1% SDS) for 1 h at 65 °C. The blots were incubated on Kodak X-Ray paper (20 × 30 cm; Kodak, Germany) for 2–4 weeks at −80 °C to visualize the phospholipase C isoenzymes.

Fluid shear flow. For fluid shear flow experiments, cells were plated three days prior to the experiment on coverglasses (Marienfeld 24 × 60 mm, No. 1) with a density of 4000 cells/cm^2. Osteosarcoma cells were cultivated in RPMI 1640 medium (Life Technologies) supplemented with 2 mM glutamine (Life Technologies) and 10% fetal calf serum (Seromed, Germany) in a 5% CO_2 incubator at 37 °C. On the day of experimentation, the cells were incubated with 3 μM Fura-2 AM (Molecular Probes, Cat. No. F-1221), a calcium sensitive ratiometric dye, for 1 h at 25 °C in RPMI 1640 medium without FCS. After washing with PBS (Life Technologies), the coverglasses were installed in a parallel shear stress flow chamber [27].

Flow was generated by a perfusion pump (Braun, Germany) creating a steady flow. The cells were observed with a Nikon Diaphot inverted fluorescent microscope equipped with a 100 W xenon lamp. Alternate 510 nm emission images from 340/380 nm excitations were obtained with a Photonic Science Extended ISIS (Photonic science, UK) at 40 ms per alternate image and averaged over 1 s, digitized, and processed using a Quanticell 700 m imaging system (Visitech International, UK). Medium used in the experiments was either RPMI 1640 without FCS or RPMI 1640 with 2% FCS, each with an addition of 10 mM Hepes.

In the experiments, a basal flow of 12 ml/h (which generated a shear stress of 3 dyn/cm^2 at the region of observation) was maintained for 30 min after mounting the 1 mm thick glass slides. Five minutes after starting the observation with the imaging system, the flow was increased to 120 ml/h, creating a shear stress of 30 dyn/cm^2. This flow was sustained for 5 min and then downregulated to basal flow, observation continuing for further 5 min.

Statistics. Data are presented as mean values ±SEM. MTT-test was analyzed using Wilcoxon–Mann–Whitney "two sample test" [28]. A probability level of ⩽5% was accepted as significant in all analyses.

Results

To investigate the mechanosensitivity of different human osteosarcoma cell lines and primary human osteoblasts (HOB), we used a 4-point-bending machine by Jones et al. [3] to apply mechanical forces to the cells MG-63, MNNG/HOS, U-2/OS, SaOS-2, OST, and HOB.

The MTT-assay revealed a significant increase in the proliferation of MG-63 cells ($25.6 \pm 7.9\%$, $p < 0.05$) and primary human osteoblasts ($20.2 \pm 4.7\%$, $p < 0.05$) after three times bending for 30 cycles with 1 Hz and 4000 με. But there was no significant in change in the proliferation of MNNG/HOS, SaOS-2, OST, and U-2/OS (Fig. 1).

The expression analysis of PLC in the cell lines MG-63, MNNG/HOS, OST, U-2/OS, and SaOS-2 revealed a picture of interest. By Western blot analysis the expression of PLC isoenzymes β1, β3, γ1, γ2, and δ1 was detectable in all cells investigated (Fig. 2). But PLC β2 was only expressed in MG-63 and MNNG/HOS (Fig. 2), both of which increased the proliferation rate when mechanically stimulated. These results were confirmed by Northern blot (Fig. 3). Interestingly, PLC γ2 was detectable in all cells by Western blot analysis but not in OST, SaOS-2, and U-2/OS in the Northern blot analysis (Figs. 2 and 3).

We found that MG-63 cells are mechano-sensitive (Fig. 1) and PLC β2 is expressed in these cells (Fig. 2). In contrast, U-2/OS cells did not respond to mechanical

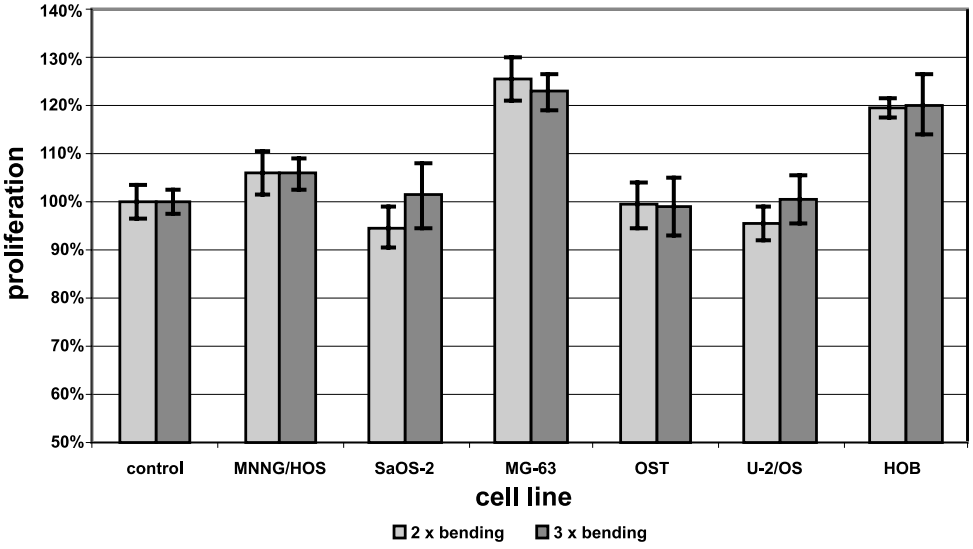

Fig. 1. MTT-test represents activity of cell lines MG-63, MNNG/HOS, SaOS-2, OST, U-2/OS, and primary human osteoblasts (HOB) after mechanical loading using a 4-point-bending machine. Data represent mean values ± SEM ($n = 8$).

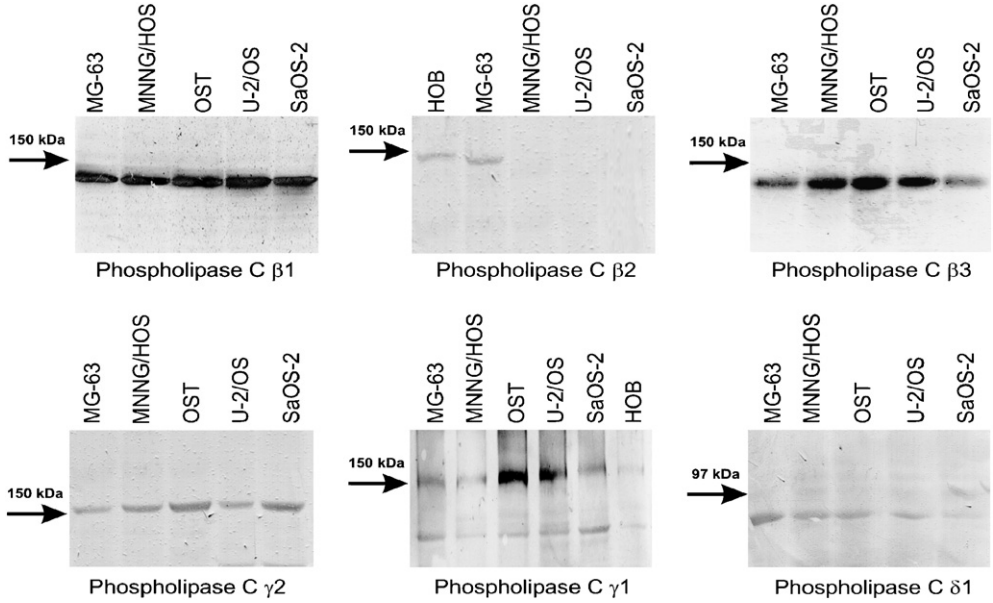

Fig. 2. Western blot analysis of the expression pattern of different isoforms of phospholipase C in human osteoblast-like osteosarcoma cells MG-63, MNNG/HOS, OST, U-2/OS, and SaOS-2 ($n = 3$).

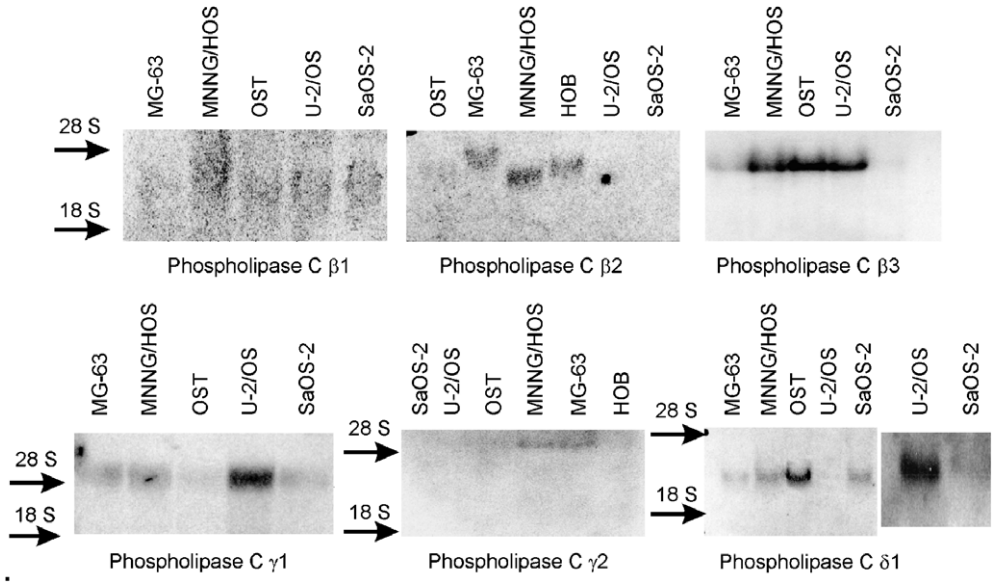

Fig. 3. Northern blot analysis of the expression pattern of different isoforms of phospholipase C in human osteoblast-like osteosarcoma cells MG-63, MNNG/HOS, OST, U-2/OS, and SaOS-2 ($n = 3$).

stimulation (Fig. 1) and failed to express PLC β2. We employed the inducible blocking system for the expression of PLC β2 in MG-63 cells to test the hypothesis that PLC β2 could be the isoform employed by the mechanosensing system. U-2/OS cells were used as control.

To quantify the degree of knockdown of phospholipase C β1 and β2 due to antisense-expression, a Western blot analysis was carried out. Stably transfected cells were incubated for 0, 6, 24, and 48 h with 10 μM ponasterone A. Incubation of U-2/OS cells stably transfected with antisense-expression vector against PLC β1 for 0 h showed a reduction of 9% compared with U-2/OS cells, transfected with pVgRXR vector, and incubated with ponasterone A for 24 h. Longer incubation periods of U-2/OS-antisense cells revealed a 21% reduction after 6 h, 32% after 24 h. Incubation for 48 h results in an increase of 20% (Fig. 4). Ponasterone A incubation of MG-63 cells, stably transfected with PLC β2 antisense, showed a reduction of PLC β2 by 18% for 0 h incubation, 26% for 6 h, 29% for 24 h, and 10% for 48 h, compared with MG-63 cells, transfected with pVgRXR vector, and incubated with ponasterone A for 24 h (Fig. 5). However, there was no difference in PLC β1 expression levels in MG-63 cells transfected with anti-PLC β2. In U-2/OS cells stably transfected with anti-PLC β2, no change in PLC β1 expression was detectable. This indicates that the antisense were isoform-specific. The same levels of PLC β2 were found in "wild-type" MG-63 and MG-63 cells expressing antisense against PLC β1.

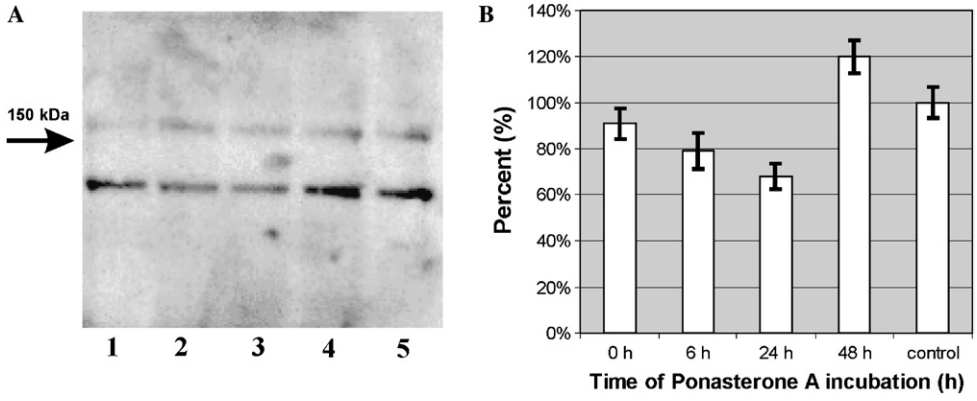

Fig. 4. Western blot analysis of phospholipase C β1. (A) The Western blot of U-2/OS cells, stably transfected with PLC β1-antisense-vector, revealed different intensities of PLC β1 expression, depending on different periods of induction with ponasterone A. Times were 0 h (1), 6 h (2), 24 h (3), and 48 h (4). Intensities were compared with stably transfected U-2/OS cells with the pVgRXR vector and incubation with the steroid hormone for 24 h (5). (B) The quantitative analysis of the Western blot with the Scion Image program of NIH. Data represent mean values ± SEM ($n = 6$).

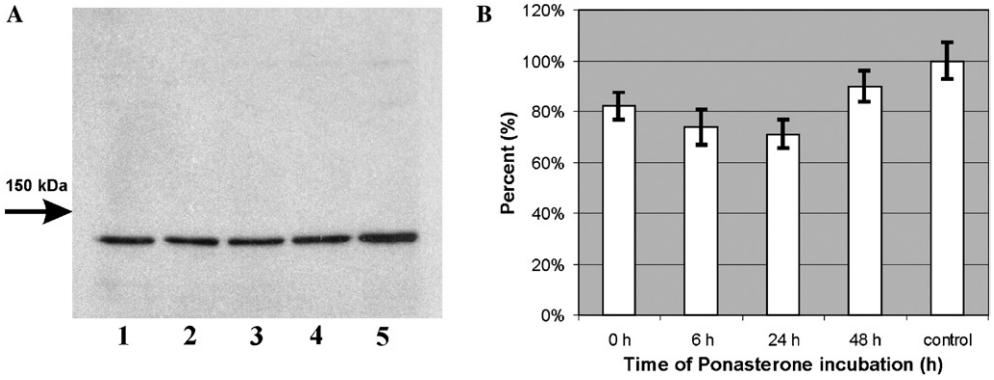

Fig. 5. Western blot analysis of phospholipase C β2. (A) MG-63 cells, stably transfected with PLC β2-antisense vector and pVgRXR vector, were probed with anti-PLCβ2-antibody after different times of incubation with ponasterone A. Periods were 0 h (1), 6 h (2), 24 h (3), and 48 h (4). As control MG-63 cells, stably transfected with pVgRXR vector and incubated with ponasterone A for 24 h, were used (5). (B) Quantitative analysis of the MG-63 Western blot. Data represent mean values ± SEM ($n = 6$).

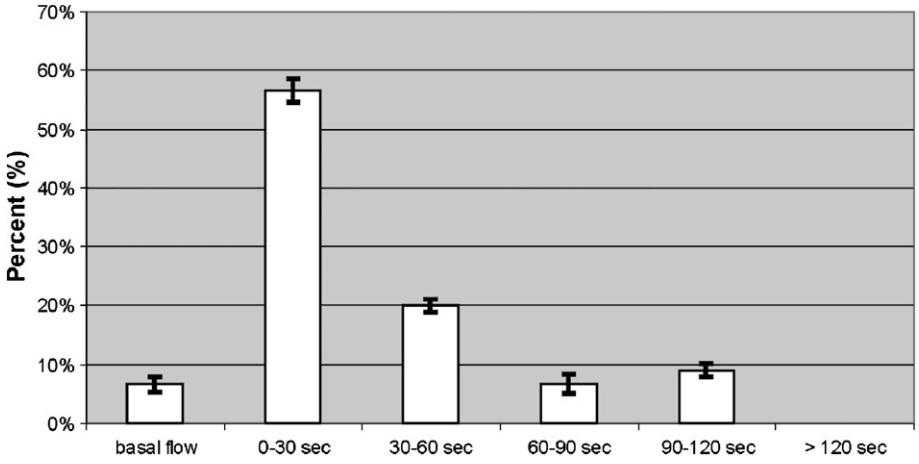

Fig. 6. Time of detachment of MG-63 cells, stably transfected with PLC β2-antisense vector and pVgRXR vector, induced by ponasterone A incubation for 24 h, and maintained for 5 min to a flow of 30 dyn/cm^2. Data represent mean values ± SEM ($n = 8$).

When exposed to fluid shear flow, 19 ± 0.7% of observed PLC β2-antisense expressing MG-63 cells lost attachment to the surface within physiological ranges of shear stress (circa 30 dyn/cm^2 [29]), while only 0.9 ± 0.1% control MG-63 osteosarcoma cells detached ($p < 0.007$). It was also observed that more than 50% of the detached cells lost attachment during the first 30 ss after increment of flow to 120 ml/h (30 dyn/cm^2, $p < 0.03$) (Fig. 6). Intracellular free calcium increased with identical percentage in MG-63 cells and MG-63 PLC β2-antisense expressing cells when using a basal flow of 3 dyn/cm^2, while calcium response in experiments without basal flow differed significantly (Fig. 7).

Discussion

Phospholipase C is an important signal transducing enzyme in all cells so far investigated. Here, we are able to show that most of the PLC isoforms known are present in primary human osteoblasts and in many human osteosarcoma cells. Various isoforms exist which are involved in coupling different external receptor signaling pathways. In osteoblasts, PLC β2 transduces signaling from parathyroid hormone (PTH), prostaglandine E2 (PGE2), and other prostanoids [30,31]. Different isoforms of PLC are distributed in the cells in characteristic areas and most of them are also present in the nuclei [10,16]. Many growth factors are activating PLC β3 by different hypertrophic stimuli in cardiomyocytes in cardio-hypertrophy, which was investigated by antisense-expression. PLC β3 expression is required for induction of immediate early genes by insulin-like growth factor-I (IGF-I) in cardio-hypertrophy [32]. Phospholipase C β2 is only expressed in mechanosensitive cells, like MG-63 and HOB. The isoform γ2 is found in all cells investigated by Western blot analysis. In contrast, in Northern blot only MG-63 and HOS/MMNG cells

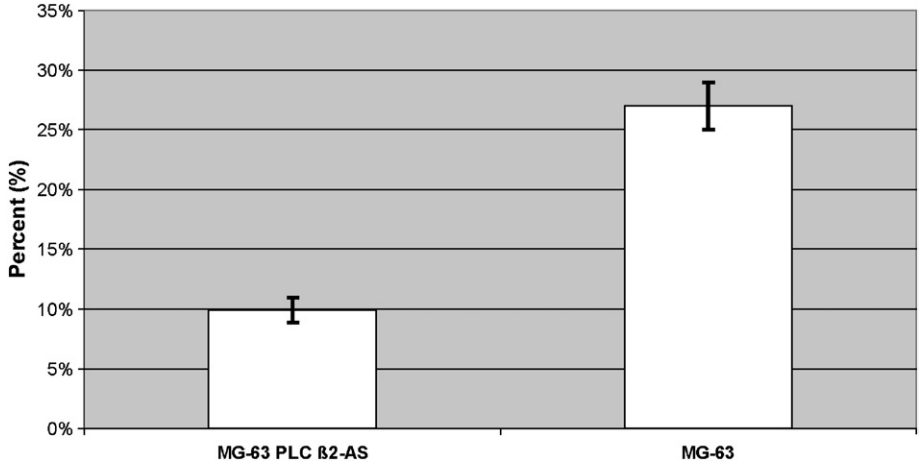

Fig. 7. Percentage of cells reacting with calcium increase after increment of flow to 30 dyn/cm^2. Data represent mean values ± SEM ($n = 8$).

showed an expression of PLC γ2. This might be because of a cross-reaction of the antibody for PLC γ2 with the protein of PLC γ1.

Nebigil [33] suppressed the individual PLC families β, γ, and δ, which resulted in a reduction of proliferation and MAP-kinase activity. However, since PLC plays such a central significant role in cell metabolism, we felt that a simply knocking out an isoform would select cells lacking a PLC that somehow compensated for the loss and were not representative of a ''normal'' cell, or might prove lethal. Hence, an inducible antisense method was chosen using the ecdysone vector expressing system. There is some discussion in the literature as to the mode of mechanical signaling, e.g., which cells are the mechano-sensing cells or whether for instance cells are, in principle, stretched or receive mechanical signals by fluid shear forces [34–39]. In analysis of the mechanisms behind mechanotransduction, it seems that the amplitudes, frequencies, and mode (uniaxial) of stretch might be equated with fluid shear flow, for which no measurements on the cell have yet been made. Similarly, it is not known whether cells on the bone surface or the osteocytes inside the bone are principally or jointly responsible for detecting the various mechanical signals. The minimal force to activate an osteoblast in cell culture is 3000 με for 30 s with 1 Hz [1]. On this culture level, most cells seem to respond to mechanical perturbations however applied by an increase of intracellular free calcium (IFC). This IFC may be generated by activation of calcium ion channel or by activation of phospholipase C [40]. A significant reduction in the number of cells with an IFC response to fluid shear flow was found in MG-63 cells expressing antisense against phospholipase C β2, but not in PLC β1-antisense expressing MG-63 cells. This suggests that there is a link between mechanotransduction and cell attachment. It is the first strong evidence for a specific role of phospholipase C β2 in signal transduction of mechanically stimulated cells.

Acknowledgments

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