Extracellular ATP stimulates the early growth response protein 1 (Egr-1) via a protein kinase C-dependent pathway in the human osteoblastic HOBIT cell line

Alex PINES*, Milena ROMANELLO*, Laura CESARATTO*, Giuseppe DAMANTE†, Luigi MORO*, Paola D'ANDREA* and Gianluca TELL*[1]

*Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università degli Studi di Trieste, via Giorgieri 1, 34127 Trieste, Italy, and Dipartimento di Scienze e Tecnologie Biomediche, Università degli Studi di Udine, P.le Kolbe 1, 33100 Udine, Italy

Extracellular nucleotides exert an important role in controlling cell physiology by activating intracellular signalling cascades. Osteoblast HOBIT cells express $P2Y_1$ and $P2Y_2$ G-protein-coupled receptors, and respond to extracellular ATP by increasing cytosolic calcium concentrations. Early growth response protein 1 (Egr-1) is a C_2H_2-zinc-finger-containing transcriptional regulator responsible for the activation of several genes involved in the control of cell proliferation and apoptosis, and is thought to have a central role in osteoblast biology. We show that ATP treatment of HOBIT cells increases Egr-1 protein levels and binding activity via a mechanism involving a Ca^{2+}-independent protein kinase C isoform. Moreover, hypotonic stress and increased medium turbulence, by inducing ATP release, result in a similar effect on Egr-1. Increased levels of Egr-1 protein expression and activity are achieved at very early times after stimulation (5 min), possibly accounting for a rapid way for changing the osteoblast gene-expression profile. A target gene for Egr-1 that is fundamental in osteoblast physiology, *COL1A2*, is up-regulated by ATP stimulation of HOBIT cells in a timescale that is compatible with that of Egr-1 activation.

INTRODUCTION

Extracellular nucleotides, such as ATP, exert stimulatory effects on eukaryotic cells via the P2 family of membrane-bound receptors [1–3]. Specific targets for nucleotides include P2Y (G-protein-coupled) and P2X (ion-channel) receptors [4]. Upon P2 receptor binding, extracellular ATP potentiates the mitogenic action of several growth factors in a variety of cell types [5,6] through the control of transcriptional activators, such as c-Fos [7]. When applied exogenously to cultured osteoblasts, ATP exerts a mitogenic action [8] and inhibits bone formation [9]; moreover, it synergizes with parathyroid hormone in stimulating c-*fos* gene expression [10].

Several cell types release ATP in response to mechanical stress or biological activation [11–13]; the peculiar mechanical sensitivity of the process has been recently highlighted by the finding that ATP release occurs in many cell types during standard experimental conditions [14]. Release of ATP and activation of P2Y receptors has been implicated as a gap-junction-independent mechanism for transducing waves of intracellular Ca^{2+} signals [15,16], and ATP can be released from osteoblasts into the local bone microenvironment via a non-lytic mechanism [7,17]. Although the possibility that locally released ATP could modulate bone-cell gene expression has been suggested [18], the actual effects and the molecular mechanisms involved have not been defined.

The early growth response protein 1 (Egr-1; also named NGFIA, TIS8, Krox-24 and Zif-268) is a member of the immediate-early transcription factors that are rapidly and transiently induced by growth factors and other signals. Several of its target genes are involved in the control of cell responses to changes in extracellular environment [19,20], and in the complex molecular processes leading to proliferation and/or differentiation (for a review, see [21]). Egr-1 contains a zinc-finger DNA-binding domain, which activates regulatory elements containing the sequence GCGGGGGCG. Such a sequence is present in a large number of genes involved in the control of cell growth and/or differentiation, such as the thymidine kinase gene [22], the collagen α2(I) gene [20,23], the tumour necrosis factor-α ('TNF-α') gene, those of the platelet-derived growth factor ('PDGF')-A, epidermal growth factor ('EGF') and insulin-like growth factor ('IGF') receptors, and the Egr-1 transcription factor itself [21], together with genes involved in the apoptotic process, such as those for Fas and Fas ligand [24]. Given its expression pattern during bone development, Egr-1 has been proposed to have a role in the biology of bone remodelling [25]. Moreover, the leading role played by Egr-1 in osteoblast physiology has also been shown by studies on redox-mediated transforming growth factor ('TGF')-β1 effects [26].

In the present paper, we show that ATP is capable of inducing both the expression and the activity of Egr-1 in the human differentiated osteoblastic cell line HOBIT. These effects are dependent upon the recruitment of a Ca^{2+}-independent protein kinase C (PKC) isoform. Notably, also mechanical stress leads to Egr-1 activation via ATP release, followed by paracrine stimulation of P2Y receptors. Interestingly, an Egr-1 target gene, collagen α2(I), is up-regulated after ATP administration. Our results point to ATP as a soluble factor responsible for Egr-1 activation in osteoblasts.

EXPERIMENTAL

Oligodeoxynucleotide synthesis and purification

Oligodeoxynucleotides were purchased from MWG-Biotech.

Abbreviations used: BAPTA-AM, bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetrakis(acetoxymethyl ester); Egr-1, early growth response protein 1; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PKC, protein kinase C; RT, reverse transcription.

[1] To whom correspondence should be addressed (e-mail tell@bbcm.univ.trieste.it).

Cell culture and materials

HOBIT cells were kindly provided by Professor B. Lawrence Riggs (Mayo Foundation, Rochester, MN, U.S.A.). ROS 17/2.8 cells were kindly provided by Professor R. Civitelli (Washington University School of Medicine, St Louis, MO, U.S.A.). SaOS-2 and MG-63 cells were kindly provided by Dr M. L. Sartori (Department of Clinical and Biological Science, University of Turin, Italy). Cells were grown in Dulbecco's minimal essential medium supplemented with 10 % (v/v) foetal calf serum, 2 mM L-glutamine and penicillin/streptomycin sulphate, and cultured at 37 °C in a humidified atmosphere containing 5 % CO_2. As the cultured cells approached confluence, they were subcultured at 1:8 splits. For the present study, cells were employed in passage number 4–9. Cell stimulation was performed on confluent cells. At 1 h before ATP stimulation, the medium was replaced by fresh medium containing the same concentrations of foetal calf serum, antibiotics and glutamine. ATP was gently mixed into a small volume of medium and subsequently added to the side of the well. The procedures for mechanical stimulation of the cells are described below. For fluorescence experiments, cells were plated on to glass coverslips.

All the chemicals described below were from Sigma–Aldrich (St Louis, MO, U.S.A.).

Nuclear extract preparation

Cell nuclear extracts were prepared as described previously [27]. Briefly, 10^7 cells were washed once with PBS and resuspended in 100 μl of hypotonic lysis buffer A [10 mM Hepes/NaOH (pH 7.9)/10 mM KCl/0.1 mM $MgCl_2$/0.1 mM EDTA/2 μg/ml leupeptin/2 μg/ml pepstatin/0.5 mM PMSF]. After 10 min, cells were homogenized by 10 strokes with a loose-fitting Dounce homogenizer. Nuclei were collected by centrifugation at 500 ***g*** and at 4 °C for 5 min in a microcentrifuge. The supernatant obtained after this centrifugation step was considered to be the cytoplasmic fraction. Nuclei were then washed 3 times with the same volumes of buffer A in order to minimize cytoplasmic contamination of the nuclear fraction. Nuclear proteins were extracted with 100 μl of buffer B [10 mM Hepes/NaOH (pH 7.9)/400 mM NaCl/1.5 mM $MgCl_2$/0.1 mM EDTA/2 μg/ml leupeptin/2 μg/ml pepstatin/0.5 mM PMSF]. After incubating for 30 min at 4 °C, samples were centrifuged at 12 000 ***g*** for 20 min at 4 °C. Nuclear extracts were then analysed for protein content [28] and stored at − 80 °C in aliquots.

Western blot analysis

The indicated amounts of nuclear extracts, obtained from HOBIT cells incubated under different conditions, were electrophoresed on to an SDS/10 % polyacrylamide gel. Proteins were then transferred on to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, U.S.A.). After transfer, membranes were saturated by an overnight incubation at 4 °C with 10 % (w/v) non-fat dried milk in PBS/0.1% (v/v) Tween 20, and then incubated with the polyclonal anti-Egr-1 antibody from Santa Cruz Biotechnology (sc-110) for 3 h. After three washes with PBS/0.1% Tween-20, membranes were incubated with an anti-rabbit immunoglobulin coupled with horseradish peroxidase (Sigma). After a 60 min incubation at room temperature, the membranes were washed several times with PBS/0.1% Tween 20, and the blots were developed using the ECL® chemiluminescence procedure (Amersham Pharmacia Biotech, Milan, Italy). Normalizations were performed using a polyclonal anti-actin antibody (Sigma). Blots were quantified by using a Gel Doc 2000 video-densitometer (Bio-Rad Laboratories).

Electrophoretic shift assay (EMSA) analysis

Double-stranded oligodeoxynucleotides, labelled at the 5′ end with [^{32}P]P_i, were used as probes in gel-retardation assays. The specific Egr-1 consensus binding site, here named *egr-1BS* oligonucleotide, is a 30-mer whose upper strand is 5′-GGATCCAGCGGGGGCGAGCGGGGGCGAACG-3′. The mutant Egr-1 binding site that was used for competitions, here named *egr-1MS* oligonucleotide, is a 30-mer whose upper strand is 5′-GGATCCAGCGGGtaCGAGCGGGtaCGAACG-3′ (the mutated nucleotides are shown underlined). Both the wild-type and mutant oligonucleotides were from Geneka Biotechnology Inc. (Montreal, Canada). The specific AP-1 binding site, here named *ap-1BS* oligonucleotide, is a 21-mer, the upper strand of which is 5′-CGCTTGATGAGTCAGCCGGAA-3′. The gel-retardation assay was performed by incubating protein and DNA in a buffer containing 20 mM Tris/HCl, pH 7.6, 75 mM KCl, 0.25 μg/ml BSA with calf-thymus DNA (25 μg/ml) (as described in the Figure legends) and 10 % glycerol for 30 min at room temperature. Protein-bound DNA and free DNA were separated on a native 5 % polyacrylamide gel run in 0.5 × TBE buffer (where 1 × TBE = 45 mM Tris/45 mM borate/1 mM EDTA) for 1.5 h at 4 °C. The gel was dried and then exposed to an X-ray film at − 80 °C. EMSA signals were quantified by using the Gel Doc 2000 videodensitometer system from Bio-Rad.

Mechanical stimulation of cells

Measurements were performed on confluent cells. Before analysis, cells were washed once with PBS: the medium was gently aspirated without tilting the plate, and PBS was gently added to the side of the well. PBS was then aspirated, and a modified Krebs solution containing 25 mM Hepes/NaOH buffer, pH 7.4, 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2 mM $CaCl_2$ and 10 mM glucose, with an osmolarity of 305 mOsM, was added as a standard extracellular solution. Particular care was taken to minimize mechanical perturbation of cells during these procedures. A medium displacement method was used as a mechanical stimulus of HOBIT cells [17]. For ATP-release measurements, one-half of the volume of the bathing medium was gently pipetted up and down twice with a micropipette. For hypotonic stress, the hypotonic solution was obtained by decreasing the concentration of NaCl to 80 mM, resulting in a solution of 215 mOsM.

Reverse transcription (RT)-PCR analysis

Total RNA was purified from HOBIT cells using the SV Total RNA Isolation System from Promega, according to the manufacturer's protocol. RT-PCR was performed with 1 μg of total RNA as the template. COL1A2 and control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were reverse-transcribed using a 20-mer oligo(dT), and then were amplified using the specific primers: COL1A2 forward (5′-TGGGAGTGCAAGGATACTCTATATCG-3′) and COL1A2 reverse (5′-CCCATCCCATCTTCGACGTAC-3′) [23], giving an amplified product of 320 bp; GAPDH forward (5′-TCTAGACGCCAGGTCAGGTCCACC-3) and GAPDH reverse (5′-CCACCCATGGCAAATTCCATGGCA-3′), giving an amplified product of 598 bp [29]; and EGR-1 forward (5′-TCCCGGCTCCTCGACCTAC-3′) and EGR-1 reverse (5′-AAGGTTGCTGTCATGTCCGAAAG-3′), giving an amplified product of 176 bp [30].

The amplified products were resolved on a 1.5 % (w/v) agarose gel.

Ca^{2+} imaging

HOBIT cells grown on to coverslips were loaded at 37 °C with fura-2 AM (5 μM), dissolved in 20 % (w/v) Pluronic (Molecular Probes, Eugene, OR, U.S.A.) (1:2 dilution), and added to the culture medium. After 60 min, the loading solution was removed and the cells were washed 3 times with a solution containing (in mM): NaCl 125, KCl 5, $MgSO_4$ 1, KH_2PO_4 0.7, $CaCl_2$ 2, glucose 6, Hepes/NaOH buffer 25, pH 7.4. Videomicroscopy and Ca^{2+} measurements were performed at 37 °C. The digital fluorescence-imaging microscopy system is built around a Zeiss inverted Axiovert 100 TV microscope (Carl Zeiss Jena, Jena, Germany). Cells were excited at 340 and 380 nm by a modified Jasco CAM-230 dual-wavelength microfluorimeter. Fluorescence images collected through a Zeiss oil immersion 40 X 1.8 NA objective were captured by a low-light-level CCD (charge-coupled-device) camera (Hamamatsu Photonics, Tokyo, Japan), and fed into a digital-image processor developed in the laboratory. Video frames were then digitized, integrated and processed off-line to convert fluorescence data into calcium maps (using the 340/380 nm excitation-wavelength-ratio method). Stimulations were performed under a continuous fluid flow of 3 ml/min. The inflow pipette was placed in the proximity (200–300 μm) of the stimulated cell (same focal plane), and an outflow port was positioned 1 mm from the stimulated cell, along the direction of the flow.

Luciferin–luciferase bioluminescence assay

The extracellular ATP concentration was determined using a kit from Molecular Probes. During the analysis, 50 μl of the luciferin–luciferase assay medium [1 mM luciferin/2.5 μg/ml luciferase/25 mM Tricine buffer (pH 7.8)/5 mM $MgSO_4$/0.1 mM EDTA/2 mM dithiothreitol] was added to 50 μl of the sample in the cuvette. The resulting light signal was immediately measured by a LB 9501 Lumat luminometer (Berthold GmbH, Bad Wildbad, Germany). A calibration curve was generated for each luciferase assay using serial dilution of an ATP standard. All reagents used to stimulate cells were tested in control experiments.

Statistical analysis

All statistical analyses were performed using either the Microsoft Excel data analysis program for Student's *t* test analysis or the statistical analysis program for ANOVA with the Scheffè multiple comparison test. $P < 0.05$ was considered statistically significant.

RESULTS

The HOBIT cell line, derived from normal adult human osteoblastic cells, retains most of the osteoblastic differentiation markers [31]. HOBIT cells respond to extracellular ATP (1–100 μM) with a biphasic increase in the cytosolic Ca^{2+} concentration [16,17].

A certain level of heterogeneity was observed in terms of cell sensitivity to the nucleotide (results not shown): a maximal dose of 100 μM was usually required for inducing calcium transients in 100 % of the cells. The initial transient, originating from Ca^{2+} release from intracellular stores, is normally followed by a decline to a plateau level, due to influx of the ion from the extracellular medium. Thapsigargin, an inhibitor of intracellular Ca^{2+} pumps that induces depletion of intracellular calcium stores, totally abolished the ATP-induced Ca^{2+} rise, suggesting that the nucleotide activates P2Y receptors and thereby ruling out a major involvement of P2X receptors in the cells' response (P. D'Andrea, unpublished work).

It has been shown previously that osteoblasts express variable levels of different purinergic receptors of the P2Y family. Similarly, HOBIT cells express $P2Y_1$ and $P2Y_2$ receptor genes, as assayed by RT-PCR analysis performed on cDNAs derived from this cell line (results not shown).

Induction of Egr-1 protein activity and expression following P2 receptor stimulation

It has been demonstrated that ATP stimulation of osteoblasts leads to nuclear signalling with the activation of the *c-fos* proto-oncogene [32]. The protein product of the c-*fos* gene constitutes, together with the product of the c-*jun* gene, the heterodimer named AP-1, which belongs to the early-activated transcription factors involved in the control of several processes leading to cell proliferation [33]. Another protein belonging to the early-activated transcription factors is Egr-1. In order to test whether the ATP-induced signalling cascade has a role in Egr-1 stimulation, a time-course experiment was performed on HOBIT cells treated with 100 μM ATP. Extracellular ATP is very rapidly degraded by ectonucleotidases present in the cell-culture medium, and therefore it must act at very early times upon cell treatment. To evaluate the timing of induction of Egr-1 protein, the time-course experiment was performed during the first 30 min after ATP treatment. At the end of the incubation, cells were collected, and nuclear extracts were tested for DNA-binding activity of Egr-1. The results shown in Figure 1(A) demonstrate that a 3–4-fold activation occurs after stimulation. A competition experiment performed with a molar excess of the unlabelled specific probe (Egr-1BS), a mutated binding site (Egr-1MS) or a nuclear factor κB ('NF-κB') binding site clearly demonstrated the specificity of the retarded complex (results not shown). Egr-1 activation can occur either because of an increased DNA-binding activity of the pre-existing protein or as a result of increased protein synthesis. In order to test whether the increase in Egr-1 DNA-binding activity was linked to an increase in protein levels, we performed a Western blot analysis on the nuclear samples used in the EMSA assays. As shown in Figure 1(B), ATP treatment promotes an increase in Egr-1 expression levels, showing that maximal induction of Egr-1 (2–3-fold over the basal level) occurred as early as 5 min after treatment, and remained at an elevated level for the duration of the experiment. The time-scale for the up-regulation of the protein levels correlates well with the activation observed in EMSA experiments, suggesting a protein neosynthesis-dependent process is involved in regulating the DNA-binding activity of Egr-1. Similar effects were also seen with lower doses of ATP (1 and 10 μM; results not shown) strengthening the biological relevance of the phenomenon.

In order to ascertain whether the observed effects of ATP are a general phenomenon important for osteoblast physiology, we performed the ATP stimulation on three other osteoblast cell lines. The human SaOS-2 (ATCC number CRL-1427) and MG-63 (ATCC number HTB-85) cell lines and ROS17/2.8 cells, a rat osteosarcoma cell line [34], were treated for 30 min with 100 μM ATP, after which nuclear extracts were assayed for Egr-1 DNA-binding activity. Results shown in Figure 1(C) show that both SaOS-2 and MG-63 cell lines responded to ATP treatment in terms of Egr-1 DNA-binding activation, whereas the ROS 17/2.8

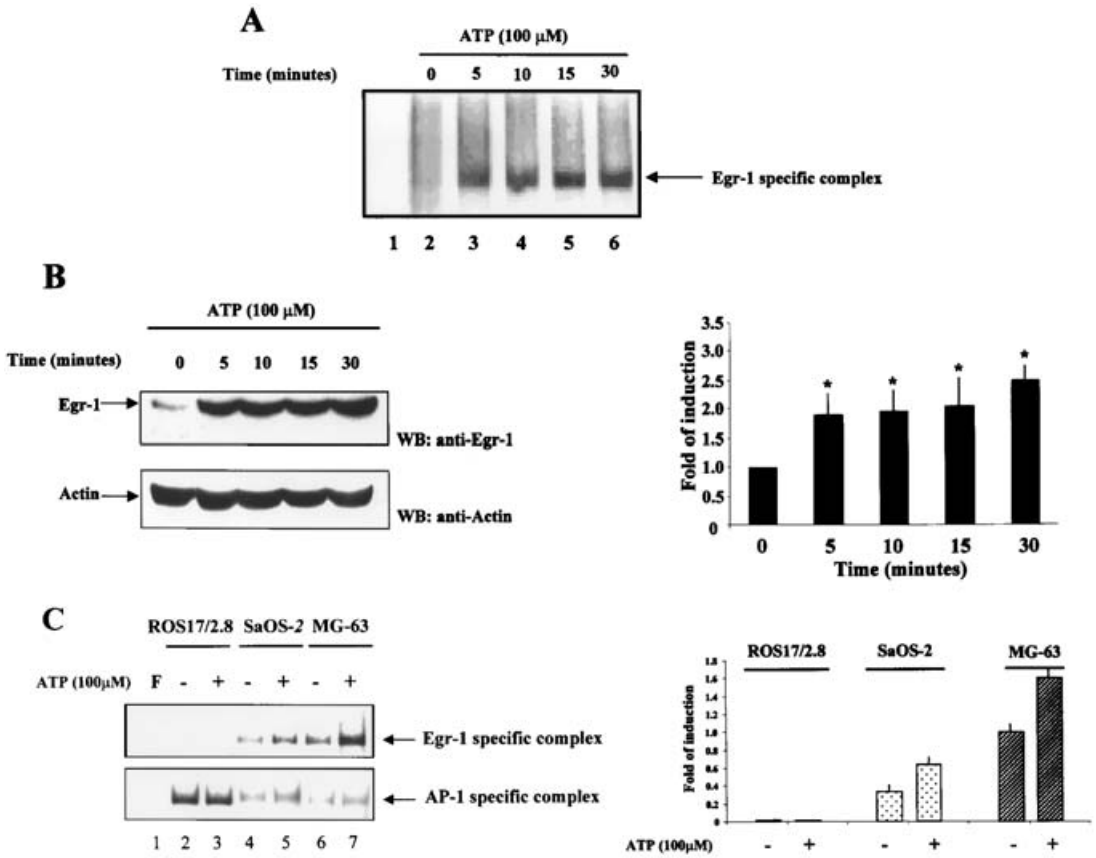

Figure 1 Stimulation of the activity and expression of the Egr-1 transcription factor at very early time-points by ATP treatment of HOBIT cells

(**A**) HOBIT cells were treated for the indicated times with 100 μM ATP. Then, cells were collected and lysed to obtain nuclear extracts that were subsequently used for EMSA analysis with the ^{32}P-labelled specific oligonucleotide called *egr-1BS* (see the Experimental section). From each nuclear extract, 5 μg was incubated with 200 fmol of labelled probe in the presence of 500 ng of calf-thymus DNA as a non-specific competitor for 30 min at room temperature, before analysis by native 5 % PAGE. After drying, the gel was exposed overnight at -80 °C for autoradiography. (**B**) Left panel: Western blot (WB) analysis of Egr-1 expression levels of samples used in EMSA assays. Nuclear extracts (15 μg) from HOBIT cells were separated on to a SDS/10 % polyacrylamide gels, blotted on to nitrocellulose membranes and assayed for the presence of Egr-1 protein by using the polyclonal anti-(Egr-1 protein) from Santa Cruz. Actin was measured as a loading control by using the anti-actin polyclonal antibody from Sigma. Right panel: values obtained from densitometric analysis of Western blot experiments, (normalized as compared with actin) are shown as histograms. Bars indicate the means $\pm$ S.D. for three independent experiments. The asterisks (*) indicate a significant difference between ATP-treated and the control ($P < 0.05$), as assessed by Student's *t* test for paired values. (**C**) Representative EMSA analysis (left panel) of Egr-1 DNA-binding activity performed by samples obtained from three different osteoblast cell lines before ($-$) and after ($+$) stimulation with 100 μM ATP for 30 min. Each nuclear extract (10 μg) was incubated with 200 fmol of labelled probe in the presence of 500 ng of calf-thymus DNA as a specific competitor for 30 min at room temperature, and then analysed by native 5 % PAGE. In order to test for the integrity of the nuclear extracts used, the DNA-binding activity of AP-1 was also assayed by using the ^{32}P-labelled specific oligonucleotide called *ap-1BS*, containing the AP-1 consensus binding site [49]. After drying, the gel was exposed overnight at -80 °C for autoradiography. Right panel: values obtained from densitometric analysis of three independent experiments are shown as histograms. Columns and bars indicate means $\pm$ S.D. respectively for three independent experiments.

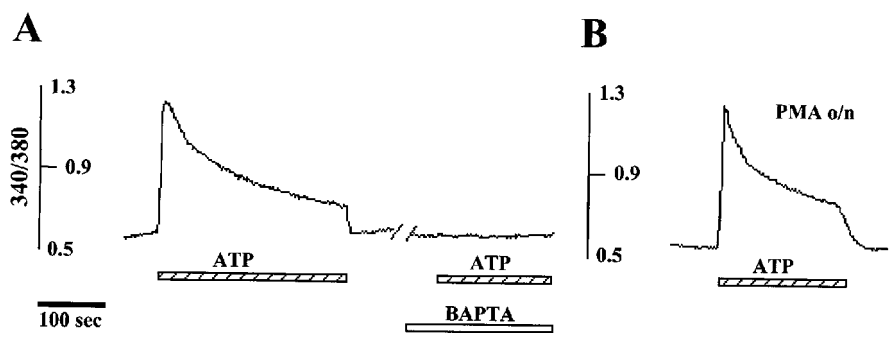

Figure 2 ATP-induced Ca^{2+} responses: effects of BAPTA-AM and PKC down-regulation

HOBIT cells loaded with fura-2 AM were analysed by digital video imaging; experiments were performed under continuous cell perfusion. (**A**) In the first part of the experiment, cells are challenged with ATP (100 μM) in the presence of 2 mM extracellular Ca^{2+}. In the second part, cells were pre-incubated with BAPTA-AM (20 μM, 1 h) and then stimulated with the same concentration of ATP. (**B**) Cells were incubated overnight (o/n) with PMA (50 ng/ml) and challenged with ATP (100 μM).

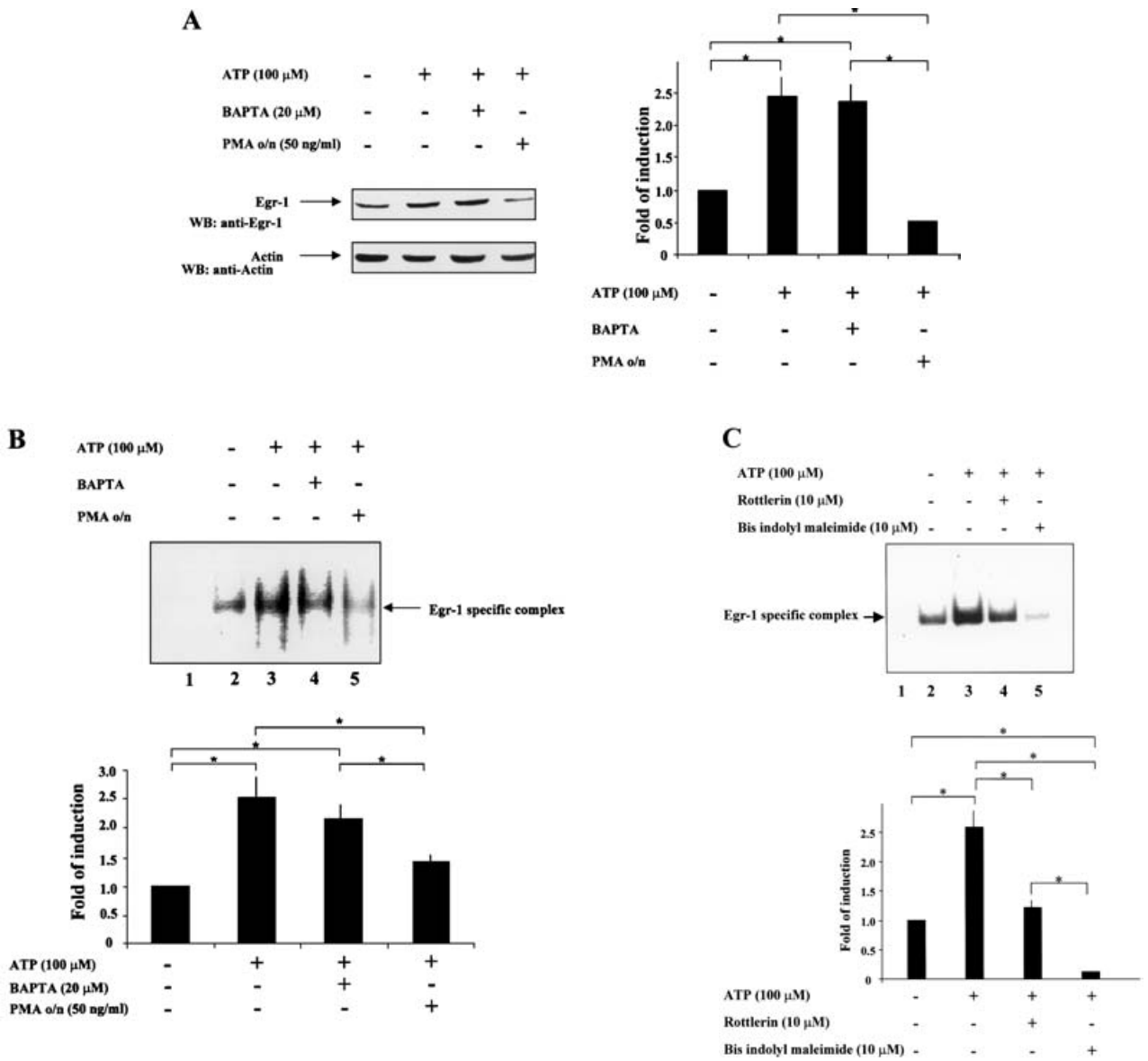

Figure 3 Activation of Egr-1 expression is dependent on PKC

(**A**) Left panel: in order to abolish the activity of PKC, HOBIT cells were pre-treated overnight with 50 ng/ml PMA. To diminish the effects of Ca^{2+} signals, cells were treated with the intracellular Ca^{2+} chelator BAPTA-AM (20 μM) for 1 h. Thereafter, cells were treated for 30 min with 100 μM ATP. Then, cells were collected and lysed to obtain nuclear extracts that were subsequently used for Western blot and EMSA analysis. Nuclear extracts (15 μg) from HOBIT cells were separated on to SDS/10 % polyacrylamide gels, blotted on to nitrocellulose membranes and assayed for the presence of Egr-1 protein by using the polyclonal anti-Egr-1 protein from Santa Cruz. Actin was measured, as a loading control, using the anti-actin polyclonal antibody from Sigma. Right panel: values obtained from densitometric analysis of three independent experiments (normalized versus actin) are shown as histograms. Columns and bars represent means $\pm$ S.D. respectively for three independent experiments (statistical analysis by ANOVA; $^*P < 0.05$). (**B**) Upper panel: the same samples used in Western blotting were assayed for DNA-binding activity by Egr-1 with EMSA analysis with the ^{32}P-labelled specific *egr-1BS* oligonucleotide (see the Experimental section). Nuclear extracts (5 μg) were incubated with 200 fmol of labelled probe in the presence of 500 ng of calf-thymus DNA as a non-specific competitor for 30 min at room temperature, and then analysed by native 5 % PAGE. Lane 1 represents the labelled oligonucleotide alone. After drying, the gel was exposed overnight at -80 °C for autoradiography. Lower panel: values obtained from densitometric analysis of three independent experiments are shown as histograms. Columns and bars indicate means $\pm$ S.D. respectively for three independent experiments (statistical analysis by ANOVA; $^*P < 0.05$). (**C**) Left panel: PKCδ isoform is responsible for the ATP-induced activation of Egr-1. In order to abolish the activity of PKCδ isoform, HOBIT cells were pre-treated for 30 min with 10 μM rottlerin. As a control for PKC involvement in Egr-1 stimulation, pre-treatment with 10 μM bis-indolylmaleimide I as a specific PKC inhibitor [50] was also performed. Thereafter, cells were treated for 30 min with 100 μM ATP. Then, cells were collected and lysed to obtain nuclear extracts that were subsequently used for EMSA analysis with the ^{32}P-labelled specific *egr-1BS* oligonucleotide (see the Experimental section). Nuclear extracts (5 μg) were incubated with 200 fmol of labelled probe in the presence of 500 ng of calf-thymus DNA as a non-specific competitor for 30 min at room temperature, and then analysed by native 5 % PAGE. Lane 1 represents the labelled oligonucleotide alone. After drying, the gel was exposed overnight at -80 °C for autoradiography. Right panel: values obtained from densitometric analysis of three independent experiments are shown as histograms. Columns and bars indicate means $\pm$ S.D. respectively for three independent experiments (statistical analysis by ANOVA; $^*P < 0.05$).

cells did not display any detectable response. This observation is of particular interest, and strengthens the importance of the role of P2Y receptors in the ATP-induced Egr-1 activation. In fact, as demonstrated previously by Jorgensen et al. [35], ROS 17/2.8 cells do not express P2Y receptors and do not respond, in terms of intracellular calcium changes, to ATP stimulation. It is noteworthy that, differently from all the osteoblast cell lines tested in the present study, ROS 17/2.8 cells do not show any detectable basal Egr-1 DNA-binding activity, even at higher concentrations of nuclear extracts (results not shown). As a control for the

quality of the nuclear extracts tested, the activity of the AP-1 transcription factor was assayed, and these results are also shown in Figure 1(C).

Activation of Egr-1 expression is dependent on a Ca^{2+}-insensitive PKC isoform

Activation of P2Y receptors leads to a simultaneous increase in concentration of two important second messengers, Ca^{2+} ions and 1,2-diacylglycerol, a physiological activator of PKC. Both Ca^{2+}-dependent and PKC-dependent pathways could stimulate Egr-1 synthesis and activity. PMA, a phorbol ester mimicking the structure of 1,2-diacylglycerol, is known to activate PKC in the short-term and to down-regulate it after long exposures [36]. Moreover, PMA is also known to activate Egr-1 in different cellular contexts, and we have observed previously that acute PMA treatment of HOBIT cells increases Egr-1 DNA-binding activity and expression levels (results not shown), suggesting that PKC could have a major role in Egr-1 activation in these cells. Interestingly, the importance of PKC activity in controlling Egr-1 expression after mechanical stress has been described in endothelial cells [37].

In order to investigate the signals responsible for ATP-induced Egr-1 up-regulation, and their possible interplay, we selectively inhibited either the Ca^{2+} signal or PKC. In calcium-imaging experiments on fura-2-loaded HOBIT cells, incubation with the intracellular Ca^{2+} chelator bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM; 20 μM for 1 h) totally prevented the ATP-induced response, indicating that this non-toxic concentration of the chelator efficiently buffered the Ca^{2+} rise (Figure 2A, $n = 3$). Lower BAPTA-AM concentrations (1–10 μM) failed to completely prevent the Ca^{2+} transient (results not shown). To investigate PKC involvement, we down-regulated the enzyme by treating the cells overnight with 50 ng/ml PMA [36]. The treatment did not affect the ATP-induced response (Figure 2B, $n = 3$), thus ruling out a major involvement of PKC in the Ca^{2+} rise. We next tested Egr-1 expression and activity in nuclear extracts obtained from cells of the same experiments. As shown in Figures 3(A) and 3(B), an overnight pre-treatment of HOBIT cells with PMA completely abolished the ATP-mediated activation of Egr-1 expression. Moreover, also the increase in the DNA-binding activity of Egr-1 is diminished after PMA treatment. Similar results were obtained with the PKC inhibitor bis-indolylmaleimide (Figure 3C, lane 5), suggesting that the ATP-mediated induction of Egr-1 in HOBIT cells is PKC-dependent. In contrast, treatment of cells with the intracellular Ca^{2+} chelator (BAPTA-AM) did not prevent the ATP-mediated Egr-1 induction, suggesting that the activation of one or more Ca^{2+}-insensitive PKC isoforms are necessarily required for Egr-1 stimulation. Indeed, specific transcripts for PKCδ and PKCε, as well as for PKCα, were detected by RT-PCR (results not shown).

In order to provide more information on which PKC isoforms are involved in the Egr-1 activation, a specific inhibitor of PKCδ activity, rottlerin, was used at a dose specific for the inhibition of this PKC isoform [38]. A 30 min pre-treatment of HOBIT cells with 10 μM rottlerin was performed before ATP stimulation. As shown in Figure 3(C), rottlerin completely prevented the activation of Egr-1 DNA binding, strongly suggesting a crucial involvement of the δ isoform in ATP-induced Egr-1 activation.

Mechanical stress induces Egr-1 activation via ATP release

Oscillatory fluid flow induces ATP release from different osteoblastic cell lines [39]. In the absence of a suitable device for generating controlled fluid flow, we have demonstrated previously that HOBIT cells release ATP under basal conditions, and in response to hypotonic stress and displacement of the medium [17]. We therefore investigated here the effects of these stimulations on ATP release, Ca^{2+} signalling and Egr-1 activation. Extracellular ATP was measured in the extracellular medium of the cells by using a sensitive luciferin–luciferase assay. To induce hypotonic stress, we incubated the cells for 10 min in a hypotonic medium (see the Experimental section). Although hypotonic stress does not correspond to a particular mechanical stress loaded on to the osteoblasts *in vivo*, hypotonic cell swelling is known to generate a stress on the cell membrane and, due to its reproducibility, has been extensively employed to study the effects of mechanical stress in other important mechanosensors, such as endothelial cells (see [40,41], and references therein). In HOBIT cells, hypotonic stress activates ATP release 2-fold compared with untreated cells (Figure 4A). As an alternative method to induce mechanical stress, we employed the so-called 'medium displacement' method (see the Experimental section), which has been employed successfully to evoke non-lytic nucleotide release from different cell types [12,17]. The efficacy of this method is probably related to an increase in bath turbulence, responsible for ATP release during standard experimental procedures [14]. Medium displacement increased the amount of ATP released by 4-fold compared with unstimulated cells (Figure 4A). Incubation of the same medium

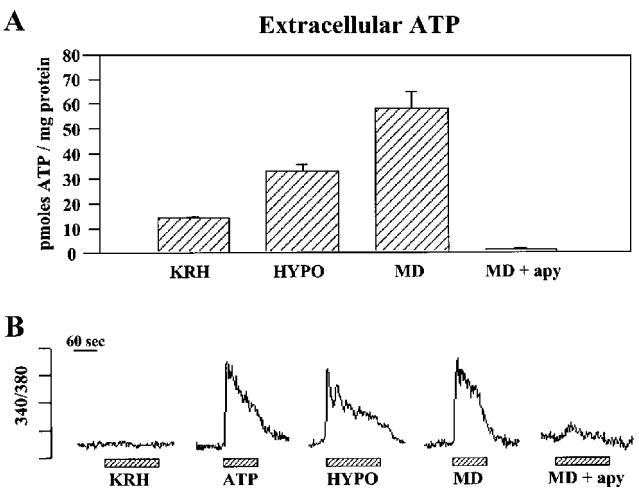

Figure 4 Effect of mechanical stimuli on ATP release and cytosolic Ca^{2+}

(**A**) ATP was evaluated in the extracellular medium of confluent HOBIT cells with a luciferin–luciferase bioluminescent assay. Release was measured 10 min after application of the solutions. Basal release (KRH) represents the amount of ATP measured in the medium of the cells maintained undisturbed in isotonic solution. Hypotonic stress (HYPO) was induced by applying an equal volume of hypotonic solution (see the Experimental section). Medium displacement (MD) was obtained by gently pipetting half the volume of the bathing medium up and down (see the Experimental section). Apyrase treatment (MD + apy) was performed by incubating the extracellular medium after medium displacement with 3 units/ml apyrase for 10 min at room temperature. The data represent the means ± S.D. from four different experiments performed in triplicate. Statistical analysis was done by ANOVA among all groups ($P < 0.05$). (**B**) Ca^{2+}-imaging recordings from HOBIT cells loaded with fura-2 AM. Experiments were performed under continuous cell perfusion. Perfusion of isotonic KRH did not induce any Ca^{2+} response. Perfusion with ATP (100 μM) induced a biphasic Ca^{2+} transient. Perfusion with hypotonic solution induced Ca^{2+} transients of the same entity (HYPO). The medium displaced from parallel confluent HOBIT cultures was collected and split into two equal amounts. The two aliquots were added either with 3 units/ml apyrase or with an equal volume of buffer, and stored for 10 min at room temperature. Perfusion of cell with untreated medium (MD) induced Ca^{2+} transients. Perfusion of fura-2 loaded cells with the medium incubated with apyrase (MD + apy) induced a slight increase of intracellular Ca^{2+} compared with the Ca^{2+} transient induced by untreated medium.

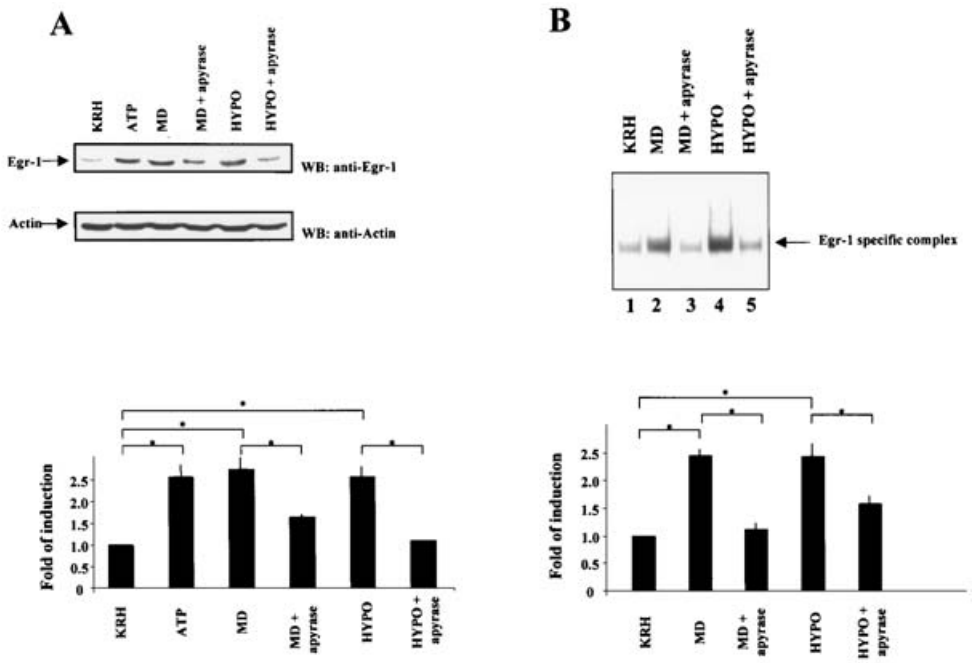

Figure 5 Induction of Egr-1 activation through ATP release by mechanical stress

HOBIT cells were mechanically stressed, as indicated in the Experimental section and in the legend to the previous Figure, and collected after 10 min to prepare nuclear extracts. Then, nuclear extracts were used for Western blot and EMSA analysis. (**A**) Upper panel: 15 μg of nuclear extracts from HOBIT cells was separated on to an SDS/10 % polyacrylamide gel, blotted on to nitrocellulose membranes and assayed for the presence of Egr-1 protein by using the polyclonal anti-Egr-1 protein from Santa Cruz. Actin was measured, as loading control, by using the anti-actin polyclonal antibody from Sigma. Lower panel: values obtained from densitometric analysis of three independent experiments (normalized versus actin) are shown as histograms. Columns and bars indicate means $\pm$ S.D. respectively for three independent experiments. (statistical analysis by ANOVA; $^*P < 0.05$). (**B**) Upper panel: the same samples used in Western blotting were assayed for DNA-binding activity by Egr-1 by EMSA analysis with the ^{32}P-labelled specific *egr-1BS* oligonucleotide (see the Experimental section). Nuclear extract (5 μg) was incubated with 200 fmol of labelled probe in the presence of 500 ng of calf-thymus DNA as a non-specific competitor for 30 min at room temperature, and then analysed by native 5 % PAGE. After drying, the gel was exposed overnight at -80 °C for autoradiography. Lower panel: values obtained from densitometric analysis of three independent experiments are shown as histograms. Columns and bars indicate means $\pm$ S.D. respectively for three independent experiments (statistical analysis by ANOVA; $^*P < 0.05$).

with 3 units/ml ATP/ADPase apyrase (for 10 min at room temperature) completely abolished the bioluminescence signal.

The ATP concentration near to the cell-membrane surface can be higher than that measured in the bulk phase, as demonstrated previously by Beigi et al. [42]. To verify whether the amount of ATP released by hypotonic stress or by medium displacement was sufficient to stimulate P2Y receptors, we performed calcium-imaging experiments on fura-2-loaded HOBIT cells. Perfusion and solution exchange of isotonic medium did not induce any changes in the cytosolic calcium concentration ($n = 5$; Figure 4B). On the other hand, hypotonic solution promptly elicited Ca^{2+} transients in 71% of the cells examined ($n = 9$; Figure 4B). Similarly to those evoked by ATP (100 μM; Figure 4B, $n = 185$), transients were followed by a sustained Ca^{2+} plateau, which returned to basal levels as cells were reperfused with the isotonic solution. Cell perfusion with the medium displaced collected from parallel cultures evoked cytosolic Ca^{2+} increases in 72 % of the cells ($n = 7$; Figure 4B). To examine whether the Ca^{2+} signal was evoked by ATP, we treated an aliquot of this medium with 3 units/ml apyrase for 10 min. Cell perfusion with apyrase-treated medium induced only a slight increase in cytosolic Ca^{2+} [70 % of the cells examined ($n = 3$); Figure 4B]. Taken together, these results demonstrated that the amount of ATP released by both hypotonic swelling and medium displacement is sufficient for activating P2Y receptors through an autocrine/paracrine loop.

In order to test whether these mechanical manipulations exert a role at the transcriptional level by triggering Egr-1 activation, HOBIT cells were stimulated either by hypotonic stress or by medium displacement. After 10 min stimulation, nuclear extracts were prepared and Western blot analysis was performed (Figure 5). Similarly to what we observed after direct administration of ATP, both hypotonic shock or medium displacement lead to an increase in Egr-1 protein levels. Moreover, pretreatment of the cells with apyrase abolishes Egr-1 activation, suggesting that ATP is the mediator responsible for the Egr-1 activation following hypotonic stress and medium displacement.

Expression of the α2(I)collagen gene after ATP treatment of HOBIT cells, and its correlation with Egr-1 activity

Type I collagen is the most abundant collagen in the bone matrix and is deposited by osteoblasts. It is composed of two identical α1(I) chains and one α2(I) chain. There is a potential Egr-1 recognition sequence conserved in the 5′ flanking region of the rat and human α2(I) collagen genes [43], and its transcription can be regulated by Egr-1 in osteoblasts [20]. In an initial attempt to verify whether the activation of Egr-1 by ATP could affect the expression of an important phenotypic marker, we tested by RT-PCR the expression levels of the α2(I) chain of type I collagen. After treatment of HOBIT cells for the indicated times with 100 μM ATP, cells were collected and RNA was prepared as described in the Experimental section. Semi-quantitative analysis by RT-PCR was then performed on the different samples with the specific primers for the α2(I) chain of type I collagen [23], and with primers for GAPDH as a control. As shown in Figure 6(A),

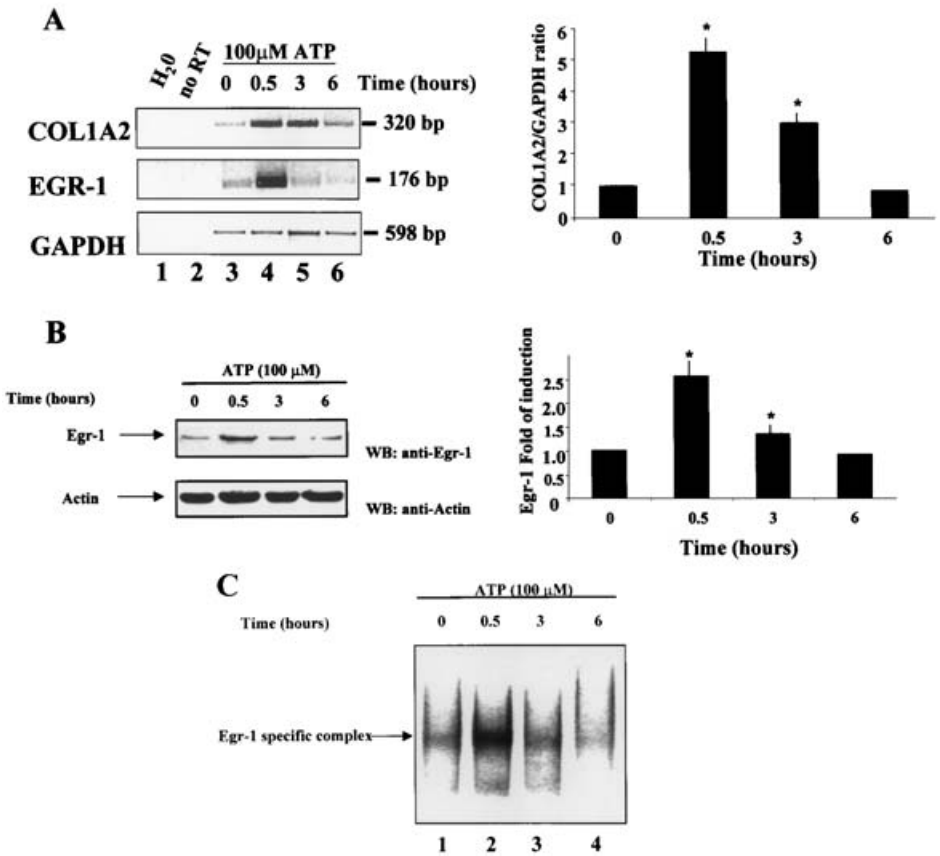

Figure 6 Induction of expression of α2(I)collagen gene in HOBIT cells by ATP treatment

(**A**) HOBIT cells were treated for the indicated times with 100 μM ATP. Then, cells were collected and RNA was prepared as described in the Experimental section. Left panel: semi-quantitative RT-PCR analysis was then performed on the *COL1A2* gene, giving a specific amplified product of 320 bp [23], and on the *EGR-1* gene giving a specific amplified product of 176 bp [30]. GAPDH was always evaluated for normalization [29]. The control reactions contained H_2O (lane 1) or no cDNA (lane 2). Right panel: values obtained from densitometric analysis of three independent RT-PCR experiments, normalized as compared with the housekeeping *GAPDH* gene, are shown as histograms. Columns and bars indicate means $\pm$ S.D. respectively for three independent experiments. *, significant difference of ATP-treated as compared with control ($P < 0.05$; Student's *t* test for paired values). (**B**) Left panel: Western blot analysis of Egr-1 expression levels of samples used in RT-PCR experiments. Nuclear extracts (15 μg) from HOBIT cells were separated on to SDS/10 % polyacrylamide gels, blotted on to nitrocellulose membranes and assayed for the presence of Egr-1 protein by using the polyclonal anti-Egr-1 protein from Santa Cruz. Actin was measured, as loading control, by using the anti-actin polyclonal antibody from Sigma. Right panel: values obtained from densitometric analysis of three independent experiments (normalized versus actin) are shown as histograms. Columns and bars indicate means $\pm$ S.D. respectively for three independent experiments. *, significant difference between ATP-treated and control ($P < 0.05$; Student's *t* test for paired values) (**C**) EMSA analysis of Egr-1 DNA-binding activity performed by samples used in Western blot assays. Nuclear extracts (5 μg) were incubated with 200 fmol of labelled probe in the presence of 500 ng of calf-thymus DNA as a non-specific competitor for 30 min at room temperature, and then analysed by native 5 % PAGE. After drying, the gel was exposed overnight at -80 °C for autoradiography.

a time-dependent up-regulation of the α2(I) chain of type I collagen gene expression is induced by treatment of the cells with ATP. Interestingly, the time-scale of the increase in α2(I) chain collagen and Egr-1 transcripts parallels the increase in Egr-1 protein (Figure 6B) and DNA-binding activity (Figure 6C).

DISCUSSION

The present study was designed to investigate whether the activation of P2 receptors by extracellular nucleotides is involved in the regulation of signal-transduction mechanisms leading to Egr-1 activation. The results demonstrate that extracellular ATP stimulates a PKC-dependent pathway, ultimately leading to the activation of the transcription factor Egr-1. Interestingly, mechanically induced ATP release appears to activate the same pathways, thus suggesting an important role for these extracellular mediators in controlling signal transduction and gene expression. Moreover, the finding that the $P2Y^{-/-}$ ROS 17/2.8 cells lack basal Egr-1 DNA binding activity suggests that purinergic receptors are required for maintaining a basal level of Egr-1 activity, and is in agreement with the findings of Ostrom et al. [14], indicating that the cellular response to ATP is a key determinant of the set-point of signal-transduction pathways.

The activation of P2Y purinoreceptors by extracellular ATP is followed by the recruitment of PKC and intracellular Ca^{2+} release. However, Egr-1 stimulation by ATP resulted in a Ca^{2+}-insensitive response, ruling out a major involvement of the Ca^{2+}-dependent forms of PKC. The PKC family of proteins comprises several isoenzymes, divided into three groups according to their structure, cofactor requirements and cellular distribution [44]. The lack of dependence on cytosolic Ca^{2+} and the inhibitory effect of rottlerin on Egr-1 activation strongly suggests a crucial involvement of PKCδ.

The preliminary evidence that the collagen α2(I) gene is rapidly activated upon ATP stimulation suggests a causal relationship between Egr-1 activation and osteoblast gene expression (Figure 7). Accordingly, the effects of ATP stimulation on the expression of osteoblastic phenotypic markers are currently under investigation. Egr-1 has been involved in the transcriptional

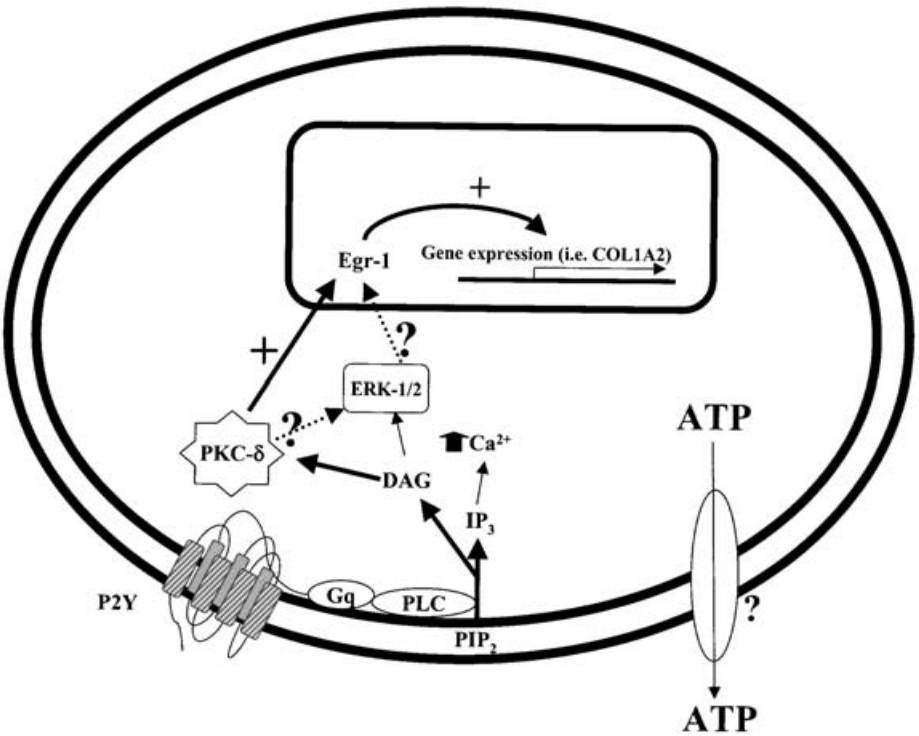

Figure 7 Model of the ATP-induced activation of Egr-1

Mechanical stress increases extracellular ATP which, in turn, stimulates *COL1A2* gene transcription via G-protein-coupled P2Y receptors and subsequent PKCδ and Egr-1-dependent signalling pathways. Signalling involving additional pathways is denoted by question marks.

regulation of the α2(I) collagen gene in osteoblasts [20,45], and PKCδ has been demonstrated recently to participate in the control of collagen gene expression in fibroblasts [46]. Taken together, these results suggest a central role for a PKC-mediated signal-transduction pathway in controlling the expression of the collagen gene in different cellular models.

The limiting step in ATP-induced cellular responses is the availability of sufficient concentrations of the nucleotide in the micro-environment surrounding the cells. ATP can be released from necrotic and apoptotic cells at sites of tissue injury, wounding or fracture, thereby reaching high local concentrations to activate P2 receptors. Nevertheless, nucleotides must exist transiently in the bone fluids without cell damage to be physiologically relevant regulators of bone remodelling. It is becoming increasingly evident that ATP release has a role in autocrine and paracrine stimulation of many cell types, including osteoblasts [16,39,47]. Mechanical stimulation in the forms of shear stress, membrane stretch or hypo-osmotic swelling stimulates ATP release from different cell types [11,48], and oscillatory fluid flow induces ATP release from different osteoblastic cell lines [39]. Mechano-sensitivity has a major role in regulating the specialized function of different tissues, including bone. However, it is not well ascertained how physical forces are linked to cellular gene expression, i.e. whether they act directly on the cytoskeletal structure, influencing transcriptional control at the nuclear level, or by means of soluble factor(s). Our results, focused on the role of extracellular ATP in controlling Egr-1 activity, suggest a role for this soluble released factor in determining this kind of control (Figure 7). Since the Egr-1 transcription factor and P2 receptors are widely expressed, and ATP release occurs in many cell types [12–14], our results imply that such a cell-signalling mechanism is largely widespread. Accordingly, when the present manuscript was in preparation, Gerasimovskaya et al. [47] clearly demonstrated that extracellular nucleotides have a role in Egr-1 stimulation in a fibroblast cell model. This kind of activation can be employed to transmit a local stimulus to neighbouring cells, thus amplifying and co-ordinating tissue responses. Owing to its fast kinetics of activation following ATP stimulation, Egr-1 appears to be an ideal candidate in controlling osteoblast sensing of environmental stimuli.

We thank Professor L. B. Riggs for the HOBIT cells, Professor R. Civitelli for the gift of ROS 17/2.8 cells, Dr M. L. Sartori for the gift of SaOS-2 and MG-63 cells lines, Professor G. Manfioletti for the use of luminometer, Dr Margherita Maioli for helpful suggestions on RT-PCR experiments and Professor Giorgio Manzini and Professor Eileen Adamson for reading the manuscript. This work was performed within the Centre of Excellence of Biocrystallography at the University of Trieste, and was supported by grants from Procter & Gamble to L. M., from the University of Trieste to G. T. and P. D'A., from MURST-PRIN 2002 to P. D'A, and from the Agenzia Spaziale Italiana to G. T.

REFERENCES

1 Boarder, M. R., Weisman, G. A., Turner, J. T. and Wilkinson, G. F. (1995) G-protein-coupled P2 purinoceptors: from molecular biology to functional responses. Trends Pharmacol. Sci. **16**, 133–139
2 Harden, T. K., Boyer, J. L. and Nicholas, R. A. (1995) P2-purinergic receptors: subtype-associated signaling responses and structure. Annu. Rev. Pharmacol. Toxicol. **35**, 541–579
3 Gordon, J. L. (1986) Extracellular ATP: effects, sources and fate. Biochem. J. **233**, 309–319
4 Harden, T., Barnard, E., Boeynaems, H. M., Burnstock, G., Dubyak, G., Hourani, S. and Insel, P. (1998) The IUPHAR Compendium of Receptor Characterization and Classification, pp. 209–217, IUPHAR Media Ltd, London
5 Huang, N., Wang, D. J. and Heppel, L. A. (1989) Extracellular ATP is a mitogen for 3T3, 3T6, and A431 cells and acts synergistically with other growth factors. Proc. Natl. Acad. Sci. U.S.A. **86**, 7904–7908

6 Tornquist, K., Ekokoski, E. and Dugue, B. (1996) Purinergic agonist ATP is a co-mitogen in thyroid FRTL-5 cells. J. Cell. Physiol. **166**, 241–248
7 Bowler, W. B., Tattersall, J. A., Hussein, R., Dixon, C. J., Cobbold, P. H. and Gallagher, J. A. (1998) A release of ATP by osteoblasts: Modulation by fluid shear forces. Bone **22** (suppl.) 3S
8 Shimegi, S. (1996) ATP and adenosine act as a mitogen for osteoblast-like cells (MC3T3-E1). Calcif. Tissue Int. **58**, 109–113
9 Jones, S. J., Gray, C., Boyde, A. and Burnstock, G. (1997) Purinergic transmitters inhibit bone formation by cultured osteoblasts. Bone **21**, 393–399
10 Buckley, K. A., Wagstaff, S. C., McKay, G., Gaw, A., Hipskind, R. A., Bilbe, G., Gallagher, J. A. and Bowler, W. B. (2001) Parathyroid hormone potentiates nucleotide-induced $[Ca^{2+}]_i$ release in rat osteoblasts independently of Gq activation or cyclic monophosphate accumulation. A mechanism for localizing systemic responses in bone. J. Biol. Chem. **276**, 9565–9571
11 Grygorczyk, R. and Hanrahan, J. W. (1997) CFTR-independent ATP release from epithelial cells triggered by mechanical stimuli. Am. J. Physiol. **272**, C1058–C1066
12 Lazarowski, E. R., Homolya, L., Boucher, R. C. and Harden, T. K. (1997) Direct demonstration of mechanically induced release of cellular UTP and its implication for uridine nucleotide receptor activation. J. Biol. Chem. **272**, 24348–24354
13 Taylor, A. L., Kudlow, B. A., Marrs, K. L., Gruenert, D. C., Guggino, W. B. and Schwiebert, E. M. (1998) Bioluminescence detection of ATP release mechanisms in epithelia. Am. J. Physiol. **275**, C1391–C1406
14 Ostrom, R. S., Gregorian, C. and Insel, P. A. (2000) Cellular release of and response to ATP as key determinants of the set-point of signal transduction pathways. J. Biol. Chem. **275**, 11735–11739
15 Osipchuk, Y. and Cahalan, M. (1992) Cell-to-cell spread of calcium signals mediated by ATP receptors in mast cells. Nature (London) **359**, 241–244
16 Romanello, M. and D'Andrea, P. (2001) Dual mechanism of intercellular communication in HOBIT osteoblastic cells: a role for gap-junctional hemichannels. J. Bone Min. Res. **16**, 1465–1476
17 Romanello, M., Pani, B., Bicego, M. and D'Andrea, P. (2001) Mechanically induced ATP release from human osteoblastic cells. Biochem. Biophys. Res. Commun. **289**, 1275–1281
18 Bowler, W. B., Buckley, K. A., Gartland, A., Hipskind, R. A., Bilbe, G. and Gallagher, J. A. (2001) Extracellular nucleotide signaling: a mechanism for integrating local and systemic responses in the activation of bone remodeling. Bone **28**, 507–512
19 Ogata, T. (1997) Fluid flow induces enhancement of the Egr-1 mRNA level in osteoblast-like cells: involvement of tyrosine kinase and serum. J. Cell Physiol. **170**, 27–34
20 Fang, M. A., Glackin, C. A., Sadhu, A. and McDougall, S. (2001) Transcriptional regulation of alpha 2(I) collagen gene expression by fibroblast growth factor-2 in MC3T3-E1 osteoblast-like cells. J. Cell Biochem. **80**, 550–559
21 Gashler, A. and Sukhatme, V. P. (1995) Early growth response protein 1 (Egr-1): prototype of a zinc-finger family of transcription factors. Progr. Nucleic Acid Res. Mol. Biol. **50**, 191–224
22 Molnar, G., Crozat, A. and Pardee, A. B. (1994) The immediate-early gene Egr-1 regulates the activity of the thymidine kinase promoter at the G0-to-G1 transition of the cell cycle. Mol. Cell. Biol. **14**, 5242–5248
23 Levin, L. G., Rudd, A., Bletsa, A. and Reisner, H. (1999) Expression of IL-8 by cells of the odontoblast layer *in vitro*. Eur. J. Oral Sci. **107**, 131–137
24 Li-Weber, M., Laur, O. and Krammer, P. H. (1999) Novel Egr/NF-AT composite sites mediate activation of the CD95 (APO-1/Fas) ligand promoter in response to T cell stimulation. Eur. J. Immunol. **29**, 3017–3027
25 McMahon, A. P., Champion, J. E., McMahon, J. A. and Sukhatme, V. P. (1990) Developmental expression of the putative transcription factor Egr-1 suggests that Egr-1 and c-*fos* are coregulated in some tissues. Development **108**, 281–287
26 Ohba, M., Shibanuma, M., Kuruoki, T. and Nose, K. (1994) Production of hydrogen peroxide by transforming growth factor-β 1 and its involvement in induction of egr-1 in mouse osteoblastic cells. J. Cell Biol. **126**, 1079–1088
27 Tell, G., Scaloni, A., Pellizzari, L., Formisano, S., Pucillo, C. and Damante, G. (1998) Redox potential controls the structure and DNA binding activity of the paired domain. J. Biol. Chem. **273**, 25062–25072
28 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254
29 Webb, B. L., Lindsay, M. A., Seybold, J., Brand, N. J., Yacoub, M. H., Haddad, E. B., Barnes, P. J., Adcock, I. M. and Giembycz, M. A. (1997) Identification of the protein kinase C isoenzymes in human lung and airways smooth muscle at the protein and mRNA level. Biochem. Pharmacol. **54**, 199–205
30 Vincenti, M. P. and Brinckerhoff, C. E. (2001) Early response genes induced in chondrocytes stimulated with the inflammatory cytokine interleukin-1β. Arthritis Res. **3**, 381–388
31 Keeting, P. E., Scott, R. E., Colvard, D. S., Anderson, M. A., Oursler, M. J., Spelsberg, T. C. and Riggs, B. L. (1992) Development and characterization of a rapidly proliferating, well-differentiated cell line derived from normal adult human osteoblast-like cells transfected with SV40 large T antigen. J. Bone Miner. Res. **7**, 127–136
32 Bowler, W. B., Dixon, C. J., Halleux, C., Maier, R., Bilbe, G., Fraser, W. D., Gallagher, J. A. and Hipskind, R. A. (1999) Signaling in human osteoblasts by extracellular nucleotides. Their weak induction of the c-fos proto-oncogene via Ca^{2+} mobilization is strongly potentiated by a parathyroid hormone/cAMP-dependent protein kinase pathway independently of mitogen-activated protein kinase. J. Biol. Chem. **274**, 14315–14324
33 Shaulian, E. and Karin, M. (2001) AP-1 in cell proliferation and survival. Oncogene **20**, 2390–2400
34 Majeska, R. J., Rodan, S. B. and Rodan, G. A. (1980) Parathyroid hormone-responsive clonal cell lines from rat osteosarcoma. Endocrinology **107**, 1494–1503
35 Jorgensen, N. R., Geist, S. T., Civitelli, R. and Steinberg, T. H. (1997) ATP- and gap junction-dependent intercellular calcium signaling in osteoblastic cells. J. Cell Biol. **139**, 497–506
36 Lu, Z., Liu, D., Hornia, A., Devonish, W., Pagano, M. and Foster, D. A. (1998) Activation of protein kinase C triggers its ubiquitination and degradation. Mol. Cell. Biol. **18**, 839–845
37 Stula, M., Orzechowski, H. D., Gschwend, S., Vetter, R., von Harsdorf, R., Dietz, R. and Paul, M. (2000) Influence of sustained mechanical stress on Egr-1 mRNA expression in cultured human endothelial cells. Mol. Cell. Biochem. **210**, 101–108
38 Gschwendt, M., Muller, H. J., Kielbassa, K., Zang, R., Kittstein, W., Rincke, G. and Marks, F. (1994) Rottlerin, a novel protein kinase inhibitor. Biochem. Biophys. Res. Commun. **199**, 93–98
39 You, J., Jacobs, C. R., Steinberg, T. H. and Donahue, H. J. (2002) P2Y purinoceptors are responsible for oscillatory fluid flow-induced intracellular calcium mobilization in osteoblastic cells. J. Biol. Chem. **277**, 48724–48729
40 Oike, M., Kimura, C., Koyama, T., Yoshikawa, M. and Ito, Y. (2000) Hypotonic stress-induced dual Ca^{2+} responses in bovine aortic endothelial cells. Am. J. Physiol. **279**, H630–H638
41 Koyama, T., Oike, M. and Ito, Y. (2001) Involvement of Rho-kinase and tyrosine kinase in hypotonic stress-induced ATP release in bovine aortic endothelial cells. J. Physiol. **532**, 759–769
42 Beigi, R., Kobatake, E., Aizawa, M. and Dubyak, G. R. (1999) Detection of local ATP release from activated platelets using cell surface-attached firefly luciferase. Am. J. Physiol. **276**, C267–C278
43 Guenette, D. K., Ritzenthaler, J. D., Foley, J., Jackson, J. D. and Smith, B. D. (1992) DNA methylation inhibits transcription of procollagen α 2(I) promoters. Biochem. J. **283**, 699–703
44 Dekker, L. V., Palmer, R. H. and Parker, P. J. (1995) The protein kinase C and protein kinase C related gene families. Curr. Opin. Struct. Biol. **5**, 396–402
45 Alexander, D., Judex, M., Meyringer, R., Weis-Klemm, M., Gay, S., Muller-Ladner, U. and Aicher, W. K. (2002) Transcription factor Egr-1 activates collagen expression in immortalized fibroblasts or fibrosarcoma cells. Biol. Chem. **383**, 1845–1853
46 Jimenez, S. A., Gaidarova, S., Saitta, B., Sandorfi, N., Herrich, D. J., Rosenbloom, J. C., Kucich, U., Abrams, W. R. and Rosenbloom, J. (2001) Role of protein kinase C-δ in the regulation of collagen gene expression in scleroderma fibroblasts. J. Clin. Invest. **108**, 1395–1403
47 Gerasimovskaya, E. V., Ahmad, S., White, C. W., Jones, P. L., Carpenter, T. C. and Stenmark, K. R. (2002) Extracellular ATP is an autocrine/paracrine regulator of hypoxia-induced adventitial fibroblast growth: signaling through extracellular signal-regulated kinase-1/2 and the Egr-1 transcription factor. J. Biol. Chem. **277**, 44638–44650
48 Sabirov, R. Z., Dutta, A. K. and Okada, Y. (2001) Volume-dependent ATP-conductive large-conductance anion channel as a pathway for swelling-induced ATP release. J. Gen. Physiol. **118**, 251–266
49 Roman, J., Colell, A., Blasco, C., Cavalleria, J., Pares, A., Rodes, J. and Fernandez-Chec, J. C. (1999) Differential role of ethanol and acetaldehyde in the induction of oxidative stress in HEP G2 cells: effect on transcription factors AP-1 and NF-κB. Hepatology **30**, 1473–1480
50 Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F. et al. (1991) The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J. Biol. Chem. **266**, 15771–15781

Received 4 February 2003/24 April 2003; accepted 2 May 2003
Published as BJ Immediate Publication 2 May 2003, DOI 10.1042/BJ20030208