Bone 51 (2012) 389–400

Contents lists available at SciVerse ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

The regulation of osteoblast function and bone mineralisation by extracellular nucleotides: The role of p2x receptors

Isabel R. Orriss [a,*], Michelle L. Key [a], Andrea Brandao-Burch [a], Jessal J. Patel [a], Geoffrey Burnstock [b], Timothy R. Arnett [a]

[a] Department of Cell and Developmental Biology, University College London, London, UK
[b] Autonomic Neuroscience Centre, University College Medical School, London, UK

ARTICLE INFO

Article history:
Received 4 April 2012
Revised 6 June 2012
Accepted 9 June 2012
Available online 26 June 2012

Edited by: J. Aubin

Keywords:
Osteoblasts
ATP
P2X receptors
Mineralisation

ABSTRACT

Extracellular nucleotides, signalling through P2 receptors, regulate the function of both osteoblasts and osteoclasts. Osteoblasts are known to express multiple P2 receptor subtypes (P2X2,5,7 and $P2Y_{1,2,4,6}$), levels of which change during differentiation. ATP and UTP potently inhibit bone mineralisation in vitro, an effect mediated, at least in part, via the $P2Y_2$ receptor. We report here that primary rat osteoblasts express additional, functional P2 receptors (P2X1, P2X3, P2X4, P2X6, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$). Receptor expression changed with cellular differentiation: e.g., P2X4 receptor mRNA levels were 5-fold higher in mature, bone-forming osteoblasts, relative to immature, proliferating cells. The rank order of expression of P2 receptor mRNAs in mature osteoblasts was P2X4>>$P2Y_1$>P2X2>$P2Y_6$>P2X1>$P2Y_2$>$P2Y_4$>P2X6>P2X5>P2X7>P2X3>$P2Y_{14}$>$P2Y_{13}$>$P2Y_{12}$. Increased intracellular Ca^{2+} levels following stimulation with P2X-selective agonists indicated the presence of functional receptors. To investigate whether P2X receptors might also regulate bone formation, osteoblasts were cultured for 14 days with P2X receptor agonists. The P2X1 and P2X3 receptor agonists, α,β-meATP and β,γ-meATP inhibited bone mineralisation by 70% and 90%, respectively at 1 μM, with complete abolition at ≥25 μM; collagen production was unaffected. Bz-ATP, a P2X7 receptor agonist, reduced bone mineralisation by 70% and 99% at 10 μM and 100 μM, respectively. Osteoblast alkaline phosphatase activity was similarly inhibited by these agonists, whilst ecto-nucleotide pyrophosphatase/phosphodiesterase activity was increased. The effects of α,β-meATP and Bz-ATP were attenuated by antagonists selective for the P2X1 and P2X7 receptors, respectively. Our results show that normal osteoblasts express functional P2X receptors and that the P2X1 and P2X7 receptors negatively regulate bone mineralisation.

© 2012 Elsevier Inc. All rights reserved.

Introduction

It is widely accepted that extracellular nucleotides, signalling via the P2 receptors, participate in a wide number of biological processes in both neuronal and non-neuronal tissues (see reviews [1,2]). P2 receptors are subdivided into the P2X ligand-gated ion channels and P2Y G-protein-coupled receptors [3,4]. Currently, seven P2X receptors (P2X1-7) and eight P2Y ($P2Y_{1,2,4,6,11,12,13,14}$) receptors have been identified; each of these receptors has been cloned, characterised and displays distinct tissue expression and pharmacology [5,6]. P2 receptors respond to a range of adenine and uridine-containing nucleotides including adenosine triphosphate (ATP), adenosine diphosphate (ADP), uridine triphosphate (UTP) and uridine diphosphate (UDP).

In recent years, it has become evident that extracellular nucleotides play a significant role in bone, modulating the function of both osteoblasts and osteoclasts (see reviews [7,8]). Studies in the early 1990s demonstrated that extracellular nucleotides act on osteoblast-like cells to transiently increase Ca^{2+} and induce the formation of inositol triphosphate (IP_3) [9]; pharmacological profiles suggested the expression of $P2Y_1$- and $P2Y_2$-like receptors [10–12]. A number of laboratories have since reported the expression of multiple P2 receptor subtypes by osteoblasts [13–18]. Osteoblasts are known to express at least seven different P2 receptor subtypes (P2X2, P2X5, P2X7, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$) in a differentiation-dependent manner [17].

Functional effects of purinergic signalling on osteoblasts include increased proliferation [19], the modulation of osteoblast responses to systemic factors such as parathyroid hormone [20,21], increased

Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; α,β-meATP, α,β-methylene adenosine 5′-triphosphate; β,γ-meATP, β,γ-methylene adenosine 5′-triphosphate; Bz-ATP, 2′(3′)-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate; UTP, uridine triphosphate; UDP, uridine diphosphate; E-NTPdase, ecto-nucleoside triphosphate diphosphohydrolase; E-NPP, ecto-nucleotide pyrophosphatase/phosphodiesterase; ALP, alkaline phosphatase; NTP, nucleotide triphosphate; NDP, nucleotide diphosphate; NMP, nucleotide monophosphate; Pi, phosphate; PPi, pyrophosphate; LDH, lactate dehydrogenase.

* Corresponding author at: Department of Cell and Developmental Biology, University College London, London, WC1E 6BT, UK. Fax: +44 20 7679 7349.
E-mail address: i.orriss@ucl.ac.uk (I.R. Orriss).

8756-3282/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.bone.2012.06.013

interleukin-6 synthesis [22], induction of osteoblastic membrane blebbing [23] and the production of lipid mediators [24]. Additionally, *in vitro* studies have shown that ATP and UTP, at concentrations ≥1 μM, strongly inhibit bone mineralisation by cultured primary osteoblasts [25,26]. The potent inhibitory actions of ATP and UTP were consistent pharmacologically with mediation via the $P2Y_2$ or $P2Y_4$ receptor subtypes. Reactive blue 2, a $P2Y_4$ receptor antagonist, failed to prevent the nucleotide-induced block of mineralisation, suggesting that $P2Y_2$ receptor stimulation mediates the functional effects of ATP and UTP [26].

ATP is present in the cytoplasm of mammalian cells at concentrations between 2 and 5 mM. Following membrane damage or necrosis, all cells release ATP into the extracellular environment, which can then act in an autocrine/paracrine manner to influence local purinergic signalling. Controlled, physiological release of ATP response to gentle mechanical stimulation has also been demonstrated from numerous excitatory and non-excitatory cells including primary osteoblasts [27], osteoblast-like cell lines [28–30] and osteocyte-like MLO-Y4 cells [31,32]. Once released, nucleotides are rapidly broken down by an extracellular hydrolysis cascade. Molecular and functional characterisation has shown that there are four families of ecto-nucleotidases: (1) the E-NTPdases (ecto-nucleoside triphosphate diphosphohydrolase); (2) the E-NPPs (ecto-nucleotide pyrophosphatase/phosphodiesterase); (3) alkaline phosphatases (ALP) and, (4) ecto-5′nucleotidase [33]. Many ecto-nucleotidases have overlapping specificities. For example, E-NTPdases catalyse the reactions: NTP→NDP+phosphate (Pi) and NDP→NMP+Pi, whereas E-NPPs hydrolyse NTP→NMP+pyrophosphate (PPi) or NDP→NMP+Pi. Thus, the combined activity of these ecto-enzymes will limit the actions of extracellular nucleotides to cells within close proximity of the release site. Osteoblasts express three members of the E-NPP family, E-NPP1, E-NPP2 and E-NPP3 [26,34] and six members of the E-NTPdase family (E-NTPdase 1,2,3,4,5,6) [35]. Osteoblastic E-NPP activity is capable of generating significant concentrations of PPi *in vitro* [26]. Since PPi is a potent inhibitor of bone mineralisation [36], it is likely that nucleotide triphosphates exert a dual inhibitory action on bone mineralisation via both P2 receptor mediated signalling and direct hydrolysis to PPi [26].

The aim of this study was to (1) document in detail the expression of additional P2 receptor subtypes by osteoblasts and, (2) determine the effects of P2X receptor activation on osteoblast differentiation, maturation and function, paying particular attention to actions on mineralisation and ecto-nucleotidase activity.

Materials and methods

Reagents

All tissue culture reagents were purchased from Gibco (Paisley, UK); unless otherwise mentioned, other reagents were obtained from Sigma Aldrich (Poole, Dorset, UK). Molecular biology reagents were purchased from Invitrogen (Paisley, UK) and all primers were from MWG Biotech (Ebersberg, Germany). P2X receptor agonists and antagonists were purchased from Tocris Bioscience (Bristol, UK). P2Y primary antibodies were obtained from Alomone (Jerusalem, Israel), P2X antibodies from Roche Bioscience (Palo Alto, CA, USA) and donkey anti-rabbit Cy3-labelled secondary antibodies from Jackson Immunoresearch Laboratories (Philadelphia, USA).

Osteoblast cell culture

Primary rat osteoblast cells were obtained by sequential enzyme digestion of excised calvarial bones from 2-day-old neonatal Sprague–Dawley rats using a 3-step process (1% trypsin in PBS for 10 min; 0.2% collagenase type II in Hanks balanced salt solution (HBSS) for 30 min; 0.2% collagenase type II in HBSS for 60 min). The first two digests were discarded and the cells were resuspended in Dulbecco's modified essential medium (Gibco, Paisley, UK), supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin (complete mixture abbreviated to DMEM). Cells were cultured for 2–4 days in a humidified atmosphere of 5% CO_2–95% air at 37 °C in 75 cm^2 flasks until confluent. Upon confluence, cells were sub-cultured into 24-well trays in DMEM supplemented with 2 mM β-glycerophosphate, 50 μg/ml ascorbic acid and 10 nM dexamethasone (mixture abbreviated to "supplemented DMEM"), with half medium changes every 3 days. Osteoblasts were cultured in the presence of several P2X receptor agonists (1–100 μM α,β-meATP (α,β-methylene adenosine 5′-triphosphate), β,γ-meATP (β,γ-methylene adenosine 5′-triphosphate), Bz-ATP (2′(3′)-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate), ATP, PPi (pyrophosphate)) to determine the effect on cell proliferation, differentiation function and ecto-nucleotidase activity. Culture with P2 receptor antagonists (0.1–50 μM RO-3, NF110, NF279, NF449, A438079, KN-62, TNP-ATP, AZ10606120, 5′-BDBD) was used to determine the receptor subtypes mediating any effects. In addition, co-culture of ATP with 1–100 nM ivermectin, a positive allosteric modulator of the P2X4 receptor, was used to study any involvement of the $P2X_4$ receptor. All experiments were carefully pH-controlled because bone mineralisation is extremely sensitive to inhibition by acidosis [37].

Bone nodule formation by osteoblasts cultured in 24-well plates was measured using modifications of an assay described previously [25]. Briefly, experiments were terminated by fixing cell layers in 2% glutaraldehyde for 5 min; mineralised bone nodules were visualised by staining with alizarin red (1% solution in water) for 5 min, rinsed with 50% ethanol to remove excess stain, then air-dried. The plates were imaged at 800 dpi using a high-resolution flat-bed scanner (Epson Perfection Photo 3200). Binary images of each individual well were then subjected to automated analysis (Image J, http://rsb.info.nih.gov/ij/), using constant "threshold" and "minimum particle" levels, to determine the number and surface area of mineralised bone nodules.

Measurement of intracellular Ca^{2+}

Osteoblasts were seeded into poly-D-lysine-coated black walled, clear-bottomed 96-well trays (BD Biosciences, Oxford, UK) at a density of 10^4 cells/well and cultured for 8 days. Cells were twice washed with PBS and loaded for 30 min with the cell-permeant Fluo-4 AM (2 μM) in PBS containing 2.5% pluronic acid in 100 μl DMSO. After removal of the fluorophore loading solution, cell layers were washed twice more and 150 μl of PBS was added per well. The cell plates were then loaded into a fluorescence imaging plate reader (FLIPR, Molecular Devices, Wokingham, UK), together with a separate 96-well plate containing 10–100 μM α,β-meATP, β,γ-meATP, Bz-ATP and ATP (the latter as positive control). The FLIPR was programmed to transfer the agonists simultaneously to all 96 cell wells 30 seconds after commencement of recording; fluorescence was excited at 488 nm and emission was measured at 510–560 nm. Duration of recording was typically about 3 min.

Total RNA extraction and DNase treatment

Osteoblasts were cultured in 6-well trays for 4–14 days in basic medium (DMEM) and osteogenic medium (supplemented DMEM); at 4, 7 and 14 days total RNA was extracted from 2 wells using TRIZOL® reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Extracted RNA was treated with RNase-free DNase I (35 U/ml) for 30 min at 37 °C. The reaction was terminated by heat inactivation at 65 °C for 10 min. Total RNA was quantified spectrophotometrically by measuring absorbance at 260 nM. RNA was stored at −80 °C until amplification by qPCR.

Download English Version:

https://daneshyari.com/en/article/5890760

Download Persian Version:

https://daneshyari.com/article/5890760

Daneshyari.com