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ORIGINAL ARTICLE

UTP-induced ATP release is a fine-tuned signalling pathway in osteocytes

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Abstract Osteocytes reside as a cellular network throughout the mineralised matrix of bone and are considered the primary mechanosensors of this tissue. They sense mechanical stimulation such as fluid flow and are able to regulate osteoblast and osteoclast functions on the bone surface. Previously, we found that ATP is released load-dependently from osteocytes from the onset of mechanical stimulation. Therefore, the aim of the present study was to investigate whether and how ATP release can be evoked in osteocytes via purinergic receptor activation. ATP release was quantified by real-time determination using the luciferin-luciferase assay and the release pathway was investigated using pharmacological inhibition. The P2Y receptor profile was analysed using gene expression analysis by reverse transcription polymerase chain reaction, while functional testing was performed using measurements of intracellular calcium responses to P2 receptor agonists. These investigations demonstrated that MLO-Y4 osteocytes express functional $P2Y_2$, $P2Y_4$, $P2Y_{12}$ and $P2Y_{13}$ receptors in addition to the previously reported P2X receptors. Further, we found that osteocytes respond to nucleotides such as ATP, UTP and ADP by increasing the intracellular calcium concentration and that they release ATP dose-dependently upon stimulation with 1–10 μM UTP. In addition to this, osteocytes release large amounts of ATP upon cell rupture, which might also be a source for other nucleotides, such as UTP. These findings indicate that mechanically induced ATP signals may be propagated by P2 receptor activation and further ATP release in the osteocyte network and implicate purinergic signalling as a central signalling pathway in osteocyte mechanotransduction.

Keywords Osteocyte · ATP release · UTP · P2 receptors · Vesicles · Mechanotransduction

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Introduction

During physical activity such as walking or running, mechanical loading causes repetitive pressure gradients, which lead to flow of extracellular fluid through the lacunocanalicular network of the mineralised bone matrix [1–3]. Osteocytes are situated in these lacunae and connected by numerous dendritic processes through the canaliculi to both neighbouring osteocytes inside the bone and to the osteoblasts on the bone surface [4, 5]. Osteocytes are highly sensitive to fluid shear stress and

they seem to constitute an essential mechanosensitive signal transducing network through the bone [2, 6–8]. Osteocyte mechanotransduction is defined as a sequence of mechanosensation, translation of the mechanical signal into a biochemical signal and finally the expression or release of molecules, which regulate osteoblasts and osteoclasts [9–11]. A number of mechanosensors, such as primary cilia and integrins, have been suggested to sense mechanical loading in osteocytes. They are reported to translate mechanical stimulation into intracellular signals such as increased intracellular calcium concentration ($[Ca^{2+}]_i$) and decreased cyclic adenosine monophosphate (cAMP) levels [12, 13]. These intracellular events lead to the regulated expression of downstream signalling molecules, which affect bone formation and resorption, such as prostaglandin E_2 (PGE_2), etc [10, 11].

A growing body of data demonstrates that both bone-forming osteoblasts and bone-resorbing osteoclasts express a variety of P2 receptors and apply these in mechanically induced intercellular communication [14–19]. Furthermore, it is observed that both osteoblast and osteoclast formation and activity are regulated by nano- to micromolar concentrations of nucleotides such as ATP, ADP, UTP and UDP [20–25]. In particular, the $P2Y_2$ receptor seems to play an important role in bone. It has been shown to mediate intercellular communication between osteoblasts [14] and low concentrations of UTP have been observed to inhibit osteoblastic bone formation [25, 26]. Furthermore, $P2Y_2$ receptor knockout in mice is associated with increased bone formation [27]. Therefore, $P2Y_2$ receptors have been characterised as an "off-switch" in bone formation. The P2X7 receptor has been suggested to play an important role in bone mechanotransduction. P2X7 receptors are expressed in MLO-Y4 osteocytes and knockout of the P2X7 receptor in mice is associated with a reduced anabolic response to mechanical loading [28]. Hence, nucleotide signalling through P2 receptors plays an important role in the regulation of bone remodelling.

P2Y receptors are G-protein-coupled receptors, which are primarily activated by nucleotides such as UTP, UDP or ADP [29, 30]. The majority of P2Y receptors ($P2Y_{1–11}$) couples to $G_{q/11}$ proteins, which leads to the generation of inositol triphosphate, calcium release from intracellular stores and the activation of protein kinase C (PKC). PKC then phosphorylates target proteins such as c-Fos [30, 31], which is also observed to be upregulated in osteocyte mechanotransduction [32, 33]. In contrast, $P2Y_{12–14}$ receptors generally couples to $G_{i/o}$, which leads to the inhibition of adenylyl cyclase and a decrease in cAMP levels [30, 31].

Vesicular exocytosis, connexin 43 (Cx43) hemichannels and pannexin 1 (Panx1) channels have been shown to mediate ATP release, which can be initiated by, e.g. elevated $[Ca^{2+}]_i$ [34–39]. Mechanically induced ATP release is reported to occur by vesicular exocytosis from osteoblasts and via Cx43 from osteocytes and associates with the release of PGE_2 [36, 37]. In osteocytes, fluid flow stimulation induces not only intracellular calcium signalling, which can be significantly reduced when P2 receptors are antagonized, intracellular calcium stores are depleted or in calcium-free medium [40], but also hemichannel-mediated ATP release [37]. Furthermore, recent investigations in our laboratory demonstrated that osteocytes in fact emit acute and load-dependent ATP signals from the onset of fluid flow stimulation [41] and that they express P2X2 and P2X7 receptors, which may propagate mechanically initiated nucleotide signals in the osteocyte network. Moreover, we have shown that the acute phase of mechanically induced ATP release is mediated by vesicular exocytosis and possibly membrane channels [41].

Therefore, the aim of the present study was to investigate whether ATP release can be evoked in osteocytes by purinergic receptor activation and how it is released. Since the $P2Y_2$ receptor seems to play an important role in bone remodelling, we chose to study UTP-induced ATP release.

Methods

Cell culturing

MLO-Y4 osteocytes were cultured according to a previously published protocol [42]. In brief, cells were cultured in plates or flasks coated with 0.15 mg/ml rat tail collagen type I (BD Biosciences, Bedford, MA, USA) and all experiments were done using 80 % confluent cells seeded 2 days before with medium change the day before the experiment.

Reverse transcription PCR

MLO-Y4 derived total RNA was isolated using the RNeasy minikit and the RNase-free DNase Set (Qiagen, Copenhagen, Denmark). RNA was reverse transcribed into cDNA using the Omniscript Reverse Transcriptase Kit (Qiagen). cDNA was amplified using HotStarTaq DNA Polymerase Kit (Qiagen) and P2X receptor specific primers (Table 1). $MgCl_2$ was added to PCR reactions amplifying $P2Y_1$ and $P2Y_{12}$ receptors. PCR reactions were carried out on a thermal cycler (Eppendorf, Hamburg, Germany or Applied Biosystems (Life Technologies) Paisley, UK), according to the following PCR protocol (40 cycles): initial activation at 95 °C for 15 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. Annealing temperature differed for $P2Y_4$ (56 °C), $P2Y_6$ (55 °C) and $P2Y_{13}$ (53 °C) receptors. Amplified DNA was separated by agarose gel electrophoresis at 140 V for 30–35 min and visualized by UV using either the Syngene System and GeneSnap (v. 4) or Fujifilm LAS-4000 (P2X2 and P2X3

Table 1 Primers used for RT-PCR amplification of P2Y receptors

Receptor	Forward primer (5′→3′)	Reverse primer (5′→3′)	Product length (bp)
$P2Y_1$	aaa aac aaa act gtc acc tgc t	tgt cca ggt cat tgt aaa tca a	166
$P2Y_2$	ggg gag agt agt gta gct gat g	gaa cca gtt gtt gga tag gtg t	161
$P2Y_4$	ctc gtg ccc aac ctc ttc tt	agc agc acc atg att gtg ga	125
$P2Y_6$	ggc tat gaa ggg cag caa ga	tca gac agt gag atg aag gcg	739
$P2Y_{12}$	tat tcc cgg aga cac tca tat c	tac tgc gga tct gaa aga aaa t	231
$P2Y_{13}$	ggc cac tag atg tca cct ttt c	gat ggt ggg gtg gta act aga a	105

bp base pairs

receptors). Sterile water represented the negative control, whereas positive control RNA was obtained from BALB/cJ mouse brain, spleen and femoral bone marrow. The tissue was ground in liquid nitrogen or cut in small pieces in RNAlater using a pair of scissors and total RNA was isolated as described for MLO-Y4 samples.

Calcium imaging

Calcium imaging experiments were conducted as described previously [15]. In brief, cells were cultured on 25 mm glass coverslips for 2 to 3 days, then loaded with 5 μM fura-2/AM (Invitrogen Molecular Probes, Eugene, OR) dissolved in experiment medium (2.5 mM probenecid, 20 mM $NaHCO_3$ in α-modified MEM, pH 7.35–7.45) and experiments were carried out at 37 °C in 11 % CO_2 superfusion on a Zeiss Axiovert 35 microscope (Carl Zeiss Inc., Thornwood, NY). Cells were exposed to 0.1, 1, 10, 100 or 1,000 μM of the following nucleotide analogues: ADP (Roche, Mannheim, Germany), 2-methyl-thio-ATP (2-meSATP, Sigma, St. Louis, MO), UTP (Roche) and UDP (Sigma). Ratios of light emission were calibrated into calcium concentrations using the fura-2 Calcium Imaging Calibration Kit (Invitrogen). Calcium responses were calculated as the average calcium increase in five individual cells per experiment.

ATP release measurements

ATP release was measured using luciferin-luciferase reagent from the ATP Bioluminescence kit HS II (Roche) on a NOVOstar microplate reader (BMG Labtech, Ortenberg, Germany) according to an earlier described method [43]. In short, cells were cultured in white, clear bottom 96-well plates for 2 days. First, 50 μl sterile HCO_3^--free buffer (÷BIC) (140 mM NaCl, 1 mM $MgCl_2 \cdot 6H_2O$, 10 mM Hepes, 1.6 mM $K_2HPO_4 \cdot 3H_2O$, 0.4 mM KH_2PO_4, 1.5 mM $CaCl_2 \cdot 2H_2O$, 5 mM Glucose·H_2O, pH 7.4) was added to the wells, and cells rested at 37 °C and 5 % CO_2 for 10 min. Then, 50 μl luciferin-luciferase reagent dissolved in ÷BIC was added and cells rested 30 min in the NOVOstar before starting the experiment. The ATP release pathway was investigated using pharmacological inhibitors. Membrane channels were blocked by carbenoxolone (CBX, Sigma), Gap26 (Cx43) mimetic peptide (VCYNKSFPISHVR) (ANASpec, Fremont, CA), ^{10}panx1 mimetic peptide (WRQAAFVDSY) (ANASpec) and probenecid (Sigma). Vesicular exocytosis was challenged by bafilomycin A1 (Calbiochem (EMD Millipore) Billerica, MA), which blocks vesicular ATP loading [44]. All pharmacological inhibitors were allowed to pre-incubate for approximately 40 min before experiment start except for bafilomycin A1, which was pre-incubated for 2 h. Since UTP induced a slow kinetic response, ATP release was measured in plate mode: 1 s/cycle, for a total of 55 cycles with a cycle time of 10 s. This allowed us to measure up to six wells/cycle and resulted in a total measurement time of 9 min. Baseline luminescence was measured for ten cycles followed by the addition of UTP using the lowest speed, 100 μl/s. UTP and vehicle solutions were kept on ice in the NOVOstar throughout the experiment, but had room temperature when they were injected. Vehicle (sterile H_2O or DMSO) in working solutions were ≤1 % and did not affect cells or the assay. Each experiment represents the mean of triplicate measurements each calibrated using ATP standard curves produced at the end of the day. For the inhibitor-based assays, ATP standards were made for both vehicle and inhibitor solutions. Final reaction volume was 150 μl and ATP release was normalized to 10^6 cells by post-experimental cell counting.

To account for potential impurities of the UTP solution with ATP that might affect the luciferin-luciferase assay directly, all UTP solutions and experiment mixtures of UTP and the different inhibitors used were tested for activity in the assay. The luminescence of the experimental mix was used as background and subtracted from the relevant measurement on the wells containing cells. If unacceptably high luminescence from the UTP solution or the UTP + inhibitor solution was detected, the solution was discarded. After each experiment, cell viability was checked by visual inspection using light microscopy. Cells were checked for morphology, attachment to the bottom of the culture dish, cell numbers and other signs of viability. If signs of cell death were observed, the experiment was excluded from data analysis.

Statistical analysis

Results are presented as individual data points and means except for ATP release curves, which are presented as mean ±SEM. Significant differences between groups were determined by Kruskal–Wallis and Mann–Whitney statistical analyses using IBM SPSS statistical analysis software, version 19 (IBM, Armonk, NY). Values of $p \leq 0.05$ was considered significant, and the following notations were used in the graphs: *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

Results

Osteocytes express P2Y receptors

RT-PCR analysis was conducted to investigate the P2 receptor mRNA profile of MLO-Y4 osteocytes and demonstrated the expression of $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$ and $P2Y_{13}$ receptor mRNA, whereas it was not possible to demonstrate the expression of $P2Y_1$ receptor mRNA (Fig. 1 and Table 2).

Osteocytes respond to P2Y receptor agonists

The expression of functional P2Y receptors on the osteocyte cell surface was determined by measuring the intracellular calcium response to different nucleotide analogues. First, cells were stimulated with ATP and demonstrated an increase in $[Ca^{2+}]_i$ when stimulated with concentrations of 10 μM and above (Fig. 2a, b and Table 2), which indicate the expression of P2X1-7, $P2Y_2$ and $P2Y_4$ receptors. To further explore the expression of $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors, cells were exposed to UTP and responded with an increase in $[Ca^{2+}]_i$ when stimulated with concentration from 1 μM UTP and up (Fig. 2c, d). Finally, cells were exposed to $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptor-selective ADP and responded to 100 and 1,000 μM (Fig. 2e, f). In contrast, cells did not respond to the equally potent $P2Y_1$ receptor-selective 2-meSATP or $P2Y_6$ receptor-selective UDP (data not shown). Vehicle did not induce any calcium responses (n=5).

Cell rupture- and UTP-induced ATP release

Next, we wanted to know whether nucleotides can be released by cell rupture and nucleotide stimulation. Cell rupture-associated ATP release was studied by scratching the cells with a needle zero, one, three, five or seven times and cells demonstrated the release of large amounts of ATP depending on the number of scratches applied (n=7, p<0.001) (Fig. 3a, b).

Since, the above data indicate the expression of $P2Y_2$ and $P2Y_4$ receptors, UTP was chosen to study the nucleotide-induced ATP release potential. Cells were exposed to 0.1, 1 and 10 μM UTP, which led to slowly increasing ATP release with a calculated initial release rate of 0.5 nM/s (0.1 μM UTP), 1.0 nM/s (1 μM) and 1.2 nM/s (10 μM) (Fig. 4a). ATP release peaked after 7–8 min with a dose-dependent peak ATP release of 45 nM (0.1 μM UTP), 102 nM (1 μM) and 119 nM (10 μM), (n=5, p=0.001 between groups, Fig. 4). ATP release associated with vehicle injection corresponded to the mechanically induced ATP release at 100 μl/s, which has been observed earlier [41].

UTP-induced ATP release pathway

To study the general involvement of gap junctions/membrane channels in UTP-induced ATP release, cells were treated with CBX. 10–35 μM CBX had no significant effect on ATP release, but 75 μM CBX led to an increase in ATP release of more than fivefold (n=3, p=0.05, Fig. 5a). Control experiments in this setup demonstrated that injection of vehicle instead of UTP induced the release of a small amount of ATP, which corresponds to approximately 15 % of the UTP-induced response (Fig. 5b) and equals the mechanically induced ATP release observed at this injection speed. Addition of 75 μM CBX to this setup significantly reduced ATP release by approximately 50 % (n=3, p=0.05, Fig. 5b), as observed earlier for mechanically induced ATP release [41]. Gap26 (10–200 μM) had no effect on UTP-induced ATP release, indicating no involvement of Cx43 (n=3, Fig. 5c).

To further study the involvement of Panx1 channels, cells were treated with ^{10}panx-1 (10–200 μM), which resulted in an

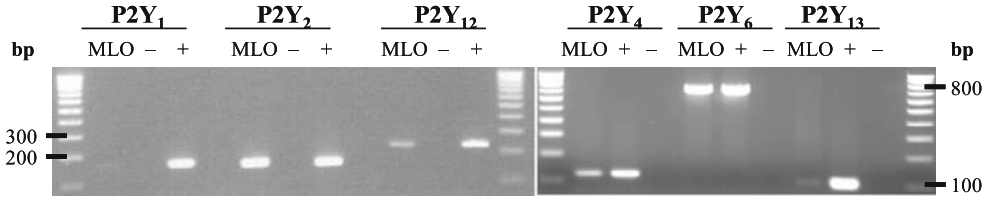

Fig. 1 P2Y receptor mRNA expression in MLO-Y4. Agarose gel electrophoresis visualizing RT-PCR amplified P2 receptor cDNA from reverse-transcribed MLO-Y4-derived RNA. The gel demonstrates the expression of $P2Y_2$, $P2Y_4$, $P2Y_{12}$ and $P2Y_{13}$ receptor mRNA in MLO-Y4 osteocytes. $P2Y_1$ receptor mRNA could not be identified. DNA marker, 100 bp DNA ladder. Positive control (+), cDNA obtained from BALB mouse bone marrow, brain and spleen. Negative control (−), sterile water. *bp* base pairs

Table 2 The P2Y receptor profile of MLO-Y4 osteocytes determined as the expression of mRNAs and functional receptors (agonist response)

Receptor	mRNA	Agonist response	Selective agonist
$P2Y_1$	−	−	ADP, 2meSATP
$P2Y_2$	+	+	ATP, UTP
$P2Y_4$	+	+	ATP, UTP
$P2Y_6$	+	−	UTP, UDP
$P2Y_{12}$	+	+	ADP
$P2Y_{13}$	+	+	ADP

The outcome of individual endpoints is indicated as positive (+) or negative (−)

average increase in ATP release of 49 %, when comparing the treated group with the vehicle group (n=3, p=0.042, Fig. 5d).

Treatment with 10–1,000 μM probenecid (n=3), a proposed Panx1 inhibitor [45], had no effect on ATP release (Fig. 5e).

UTP-induced ATP release was then challenged by bafilomycin A1. This treatment reduced ATP release by 49 % (0.5 μM, p=0.05) and 44 % (1 μM, p=0.05) (n=3, Fig. 5f), which indicates vesicular exocytosis of ATP.

After each individual experiment, cells were checked for viability by visual inspection. No signs of decreased viability/cell death were detected.

Discussion

We have previously shown that osteocytes release ATP immediately as mechanical stimulation occurs and that they

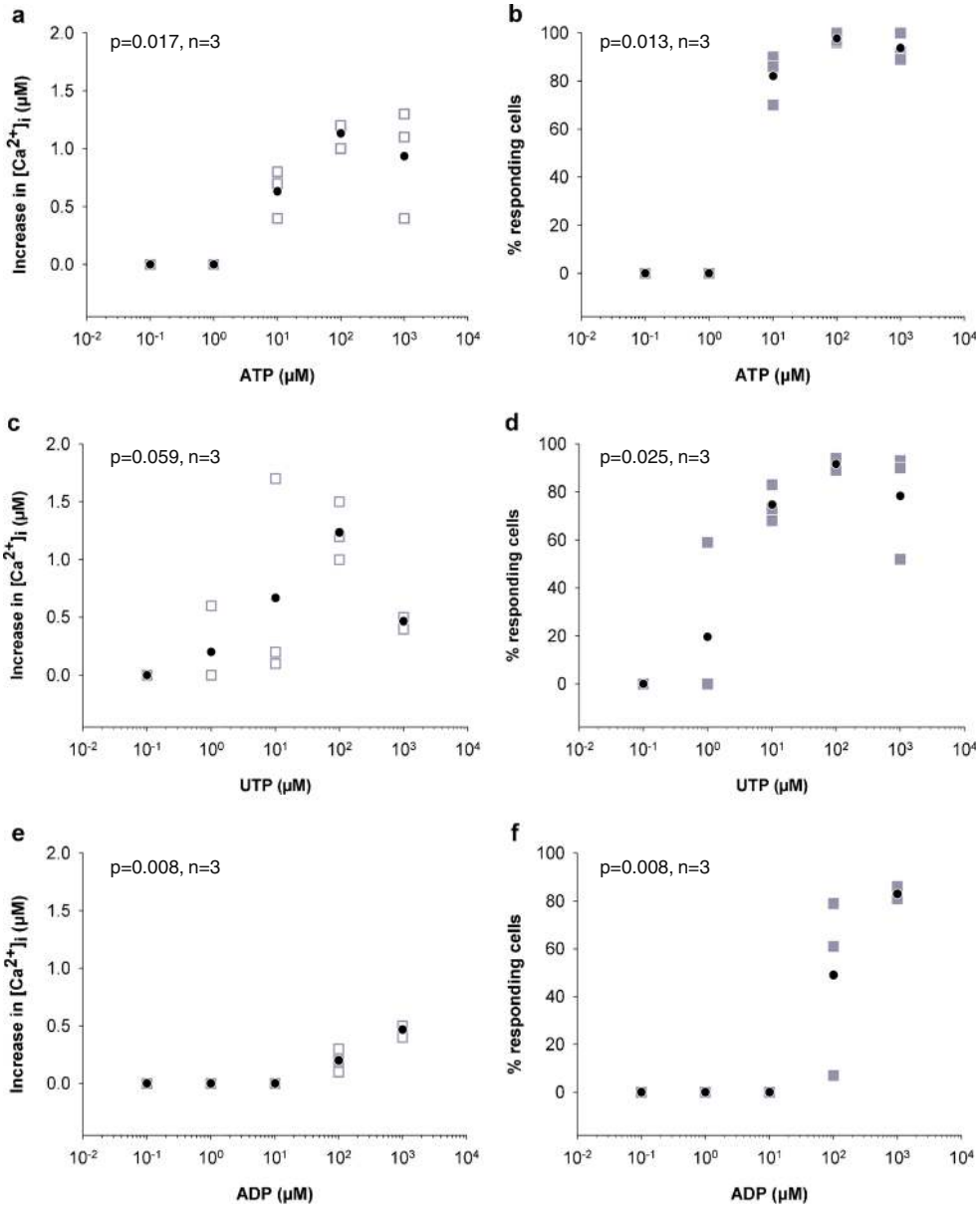

Fig. 2 Osteocytes respond to P2 receptor agonists. Nucleotide-induced intracellular calcium responses were measured in fura-2 loaded MLO-Y4 osteocytes by exposing them to 0.1, 1, 10, 100 or 1,000 μM nucleotide. MLO-Y4 responded to ATP (**a**, **b**), UTP (**c**, **d**) and ADP (**e**, **f**) by increasing $[Ca^{2+}]_i$ indicating the functional expression of $P2Y_2$ and/or $P2Y_4$, and $P2Y_{12}$ and/or $P2Y_{13}$ receptors. *Empty grey box* represents the average calcium response in each experiment, *filled grey box* represents the percent of cells responding in each individual experiment and *filled black circle* represents the mean (n=3)

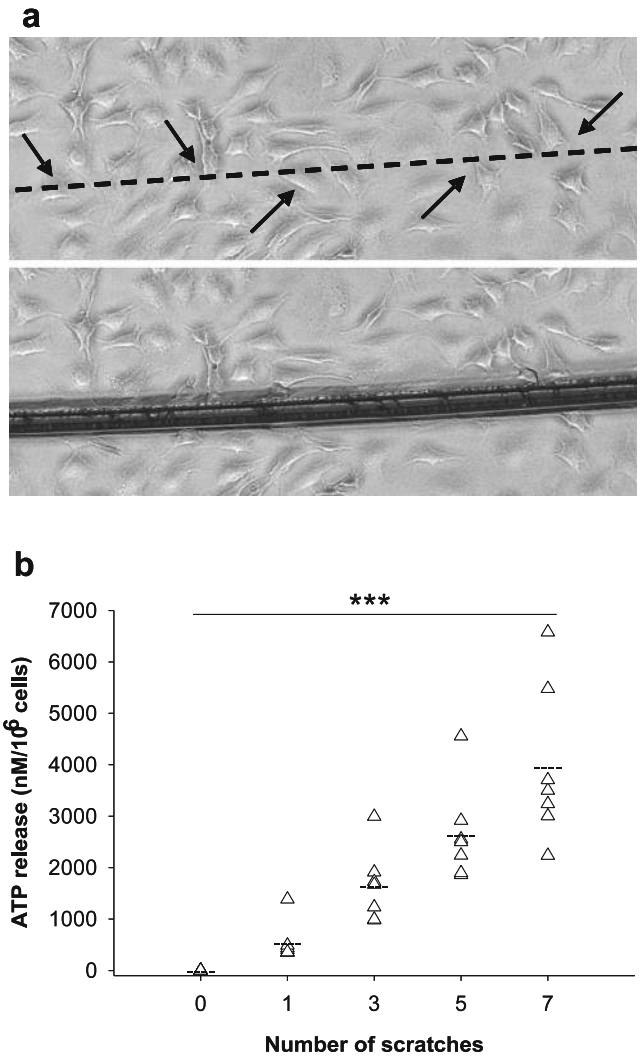

Fig. 3 Cell rupture-related ATP release. **a** Scratching the cells with a needle resulted in rupture of both cell bodies and dendrites. *Upper panel*, before scratch; *lower panel*, after scratch. **b** Cells were scratched with a needle zero, one, three, five and seven times, which led to release of ATP depending on the number of scratches (n=7). *Arrow* indicates ruptured dendrites or cell bodies, *triangle* represents ATP release in each experiment, *broken line* represents mean ATP release and *three asterisks* represent $p \leq 0.001$

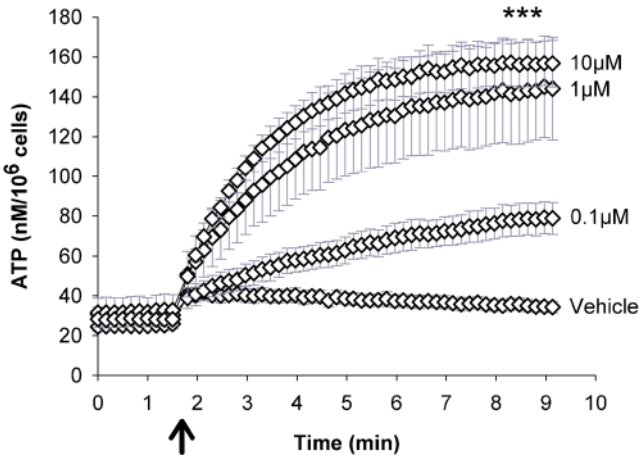

Fig. 4 UTP induced ATP release. After ten baseline measurements, UTP (0.1, 1 and 10 μM) was injected in the wells leading to slowly increasing and dose-dependent ATP release for the subsequent 7–8 min. Vehicle induced the release of a low amount of ATP, which reflect mechanically induced ATP release at the applied injection speed (n=5). ATP release induced by UTP was quantified and normalised to 10^6cells. Data are given as mean±SEM; *arrow* indicates UTP addition, *three asterisks* represent $p \leq 0.001$

express functional ATP-sensing P2X receptors. This indicates that P2 receptor-mediated ATP release may be an early signal in osteocyte mechanotransduction. In the present study, the aim therefore was to study whether osteocytes are able to respond to nucleotide stimulation by P2 receptor activation and ATP release.

First, we analysed the expression of P2Y receptors in order to find the P2 receptor profile of osteocytes. RT-PCR revealed the expression of $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$ and $P2Y_{13}$ receptor mRNA, whereas $P2Y_1$ receptor mRNA expression could not be identified. $P2Y_2$/$P2Y_4$ and $P2Y_{12}$/$P2Y_{13}$ functional receptor expression was demonstrated by UTP- and ADP-induced calcium responses, respectively. The availability of $P2Y_2$ and $P2Y_4$ receptor-selective agonists and antagonist was highly limited, which made further pharmacological distinction between these receptors unfeasible. On the human $P2Y_4$ receptor, UTP is observed to be more potent than ATP. However, on the mouse $P2Y_4$ receptor, they are equipotent [30] and pharmacological discrimination by analysing the different potencies of UTP and ATP was therefore not possible.

$P2Y_2$ receptors have been implicated in osteoblast intercellular communication and characterized as an "off-switch" in bone remodelling [14, 25, 26]. Its expression on osteocytes may also implicate it in osteocyte signalling pathways, which we investigated further by studying UTP-induced ATP release.

Discrimination between $P2Y_{12}$ and $P2Y_{13}$ receptors was similarly limited by the lack of specific agonists. $P2Y_{13}$ receptor mRNA was identified by a weak band and receptor expression may thereby be low. The ADP-induced calcium response may thereby indicate $P2Y_{12}$ receptor expression. However, quantitative analysis of P2 mRNA expression should be performed to further clarify this issue and is highly relevant. Based on the current data, $P2Y_{13}$ receptor expression cannot be excluded. In contrast, $P2Y_1$ receptor mRNA was not identified and 2-meSATP did not induce an intracellular calcium response, which indicate the complete absence of $P2Y_1$ receptors on osteocytes. Taken together, these results indicate the functional expression of $P2Y_2$ and/or $P2Y_4$, and $P2Y_{12}$ and/or $P2Y_{13}$ receptors.

The $P2Y_2$ receptor is characterised as an important P2 receptor in osteoblast communication and function [14, 25, 26]. $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors are activated by UTP, but since cells did not respond to UDP, functional $P2Y_6$ receptors

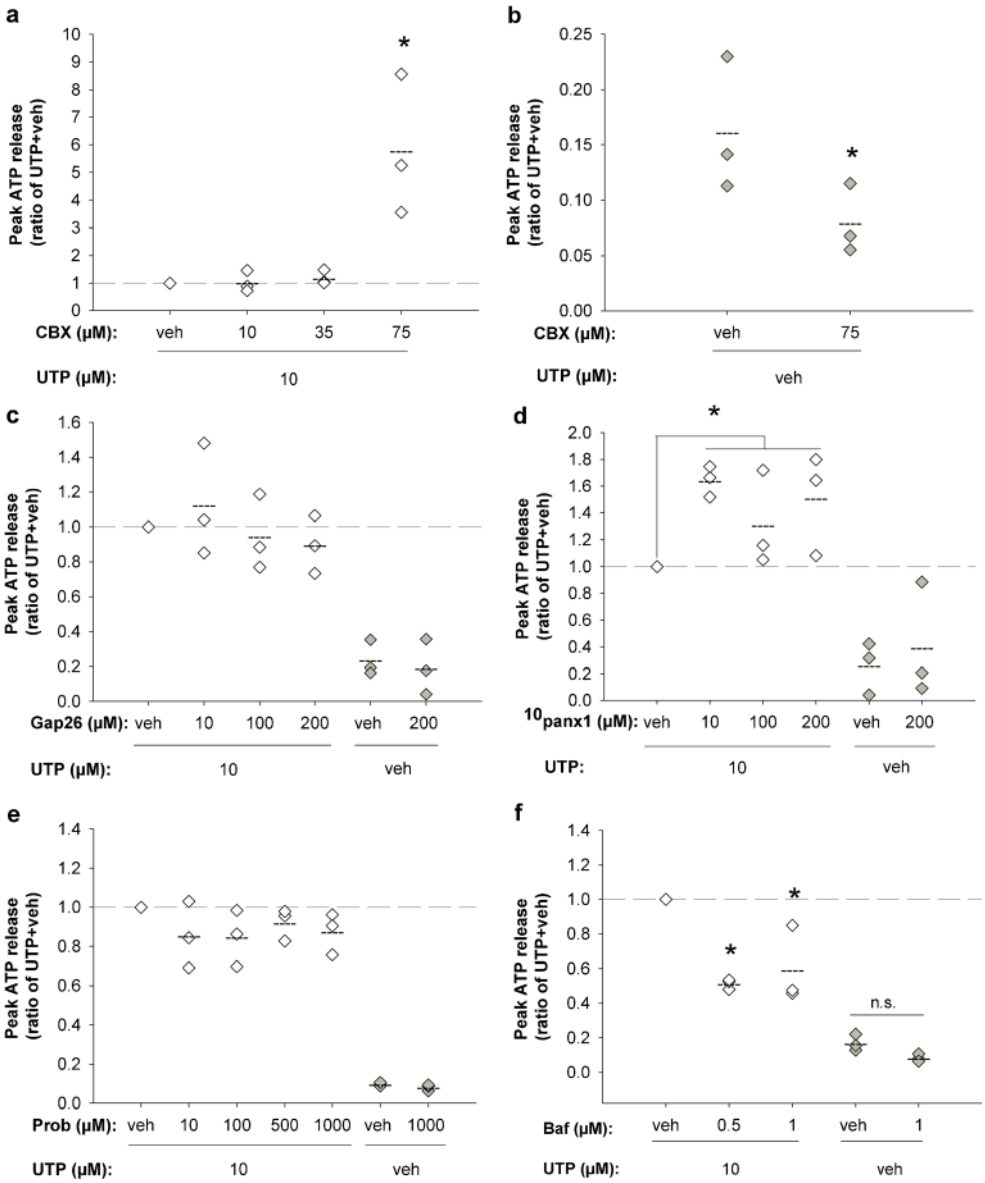

Fig. 5 Analysis of the UTP-induced ATP release pathway. ATP release induced by 10 μM UTP (100 μl/s) was challenged by pharmacological inhibitors. **a** 10–35 μM CBX had no effect on ATP release, whereas 75 μM CBX resulted in a dramatic increase in ATP release of >5-fold. **b** The addition of vehicle instead of UTP induced the release of a small amount of ATP from the cells corresponding to approximately 15 % of the UTP-induced ATP release. When 75 μM CBX was added to this setup, ATP release was reduced by approximately 50 %. **c** Gap26-mediated inhibition of Cx43 hemichannels did not affect ATP release. **d** ^{10}panx1-mediated inhibition of Panx1 channels was associated with significantly increased ATP release, when the treated group was compared to the vehicle group. **e** Probenecid (Prob)-mediated inhibition of Panx1 channels did not affect ATP release. **f** Bafilomycin A1 (Baf) treatment resulted in up to 49 % reduction of UTP induced ATP release. *Diamond* represents ATP release in ratios of the control group (UTP + vehicle of the inhibitor), *broken line* represents mean ATP release in ratio of vehicle and *asterisk* represents $p \leq 0.05$

seems to be absent. Thus, the induction of ATP release by UTP occurs via the activation of $P2Y_2$ and/or $P2Y_4$ receptors. Various sources exist for UTP in the extracellular milieu. Constitutive as well as mechanically induced UTP release has been observed in a number of cell types [46, 47]. Moreover, UTP can be generated extracellularly from the nucleoside diphosphokinase-catalysed transphosphorylation of UDP by ATP [48]. However, another potential source of high extracellular nucleotide concentrations is cell death or cell rupture and tissue damage. In bone, microfractures occur frequently throughout the skeleton and one of the functions of the remodelling process is repair these to maintain a strong and durable skeleton. This process involves the initiation of osteoclastic bone resorption and subsequently osteoblastic bone formation at the bone multicellular unit. A microfracture involves rupture of osteocytes that are embedded in the bone matrix. Our data from the scratch-wound assay showed that cell rupture of the osteocytic cells resulted in significant amounts of ATP being released. The implications of this could be that not only mechanical stimulation of the cells could induce nucleotide-induced ATP release but also cell rupture occurring in relation to microfractures in the bone tissue could initiate ATP release from neighboring osteocytes and potentially propagation of the signal to the bone surface where the ATP could activate osteoclasts and initiate the remodelling process.

Using functional studies, we showed that UTP in concentrations ≥1 μM induces intracellular calcium responses and initiates ATP release from osteocytes. Moreover, when UTP concentration was increased, ATP release increased dose-dependently. This indicates that osteocytes can translate UTP signals into ATP release and that they are able to

communicate the strength of the initial UTP signal to neighbouring cells by graded ATP release. Thus, osteocytes seem to be able to communicate fine-tuned nucleotide messages in the network.

However, since ATP release was quantified 7–8 min after stimulation, it must be considered a mixed response to first UTP stimulation and then nucleotide autocrine stimulation from released ATP (and possibly other nucleotides). Therefore, the entire response may be a result of more general P2 receptor activation initiated by UTP and potentially reflects the propagation and/or potentiation of ATP signals by auto/paracrine stimulation in the culture. The fact that UTP-stimulated P2Y receptor activation induces nucleotide release and subsequent positive feedback activation of other P2 receptors implies that the system, at least theoretically, may function as an on–off switch that can keep the system in either the on or off position. This way activation of P2Y receptors in one area of the osteocyte network can be activated, for example by microfractures with release of nucleotides from dying osteocytes or by mechanical loading on the bone and initiate an ATP-mediated P2 purinergic signalling cascade through the osteocyte canalicular network reinforcing the signal along the osteocyte dendrites until the signal reaches the bone surface. As the peridendritic space is narrow and thus the layer of extracellular fluid thin, high concentrations of ATP can be obtained around the osteocytes making this a highly efficient signalling system within the bone tissue.

The involvement of specific P2 receptor subtypes in UTP-induced ATP release may be further investigated by selective antagonists or by using cells from knockout animals. UTP release and its extracellular generation have not been investigated in osteocytes, but based on the present results, these investigations are highly relevant. The involvement of $P2Y_2$ and $P2Y_4$ receptors in the mediation of nucleotide signals in the osteocyte network is novel information, which can be applied in the analysis of bone phenotypes of P2 receptor knockout animals. $P2Y_2$ receptors are involved in osteoblast intercellular communication and its activation has been observed to reduce bone formation, thereby functioning as an off-switch in bone remodelling [14, 25, 26]. $P2Y_2$ receptor knockout animals are observed to have increased bone mass possibly because of blocked inhibition of bone formation. The present data indicate that $P2Y_2$ receptors are also involved in the mediation of nucleotide signals in the osteocyte network; thus, increased bone mass in $P2Y_2$ receptor knockout mice might also be caused by inhibited osteocyte "off-signalling" to osteoblasts. Nonetheless, it is highly relevant to study $P2Y_2$ receptor signalling in osteocytes further and how it affects bone remodelling.

Since we also found the expression of functional $P2Y_{12}$ receptors on osteocytes, they may also be involved in osteocyte intercellular communication. In fact, decreased intracellular cAMP levels have been observed as a response to mechanical stimulation of osteocytes and the same intracellular event occurs in $P2Y_{12}$ receptor-expressing cells upon $P2Y_{12}$ receptor activation [13, 29, 30]. In osteoblasts and osteoclasts, $P2Y_{12}$ receptor antagonism inhibits their formation and viability and additionally decrease bone formation and resorption in vitro. However, despite the fact that both bone formation and resorption is affected, the overall effect of $P2Y_{12}$ receptor antagonism in vivo is decreased bone mineral density and trabecular bone volume and number [49]. If $P2Y_{12}$ receptors are additionally involved in osteocyte signalling, this provides a third dimension to the analysis of $P2Y_{12}$ receptor antagonism in vivo and could explain why the overall bone phenotype is negatively affected. Flow-induced mechanosensing via primary cilia in osteocytes associates with an immediate decrease in cAMP levels and later compensatory increase in cAMP levels in addition to increased COX-2 expression [13], of which the latter is involved in the synthesis of the bone anabolic PGE_2. The $P2Y_{12}$ receptor-associated decrease in cAMP levels may thereby also lead to the release of bone formation-promoting signals such as PGE_2 from the osteocyte, which would be blocked by $P2Y_{12}$ receptor antagonism. Thus, $P2Y_{12}$ receptor antagonism in vivo may result in an overall negatively affected bone homeostasis due to blocked $P2Y_{12}$ receptor signalling in both osteocytes and osteoblasts.

Next, we challenged ATP release by pharmacological inhibitors to identify the involved release pathway. We previously found that mechanically induced ATP release could be inhibited by bafilomycin A1 [41] and here we find that 0.5 μM bafilomycin inhibits UTP-induced ATP release by 49 %. ATP release could not be further inhibited by increasing concentration to 1 μM, indicating full inhibition of the vesicular H^+-pump and supposedly the accumulation of nucleotides in intracellular vesicles. Thus, alternative ATP release pathways to vesicular exocytosis may exist in osteocytes and the release pathway was further challenged by targeting membrane channels.

Previously, we found that 35 μM CBX significantly inhibited mechanically induced ATP release [41]. In the present study, it did not affect UTP-induced ATP release. Since both Cx43 hemichannels and Panx1 channels are blocked by low micromolar concentrations of CBX [50, 51], membrane channels are not likely to be involved in UTP-induced ATP release.

Most unexpectedly, however, when CBX concentration was increased to 75 μM, ATP release was observed to increase dramatically. Since this effect was not observed in control measurements of vehicle addition, it seems to be UTP/$P2Y_{2/4}$ receptor-dependent. UTP-induced enhanced ATP release was also observed in the presence of ^{10}panx1, but not in the presence of the less potent Panx1 inhibitor, probenecid. CBX- and ^{10}panx1-based experiments thus indicate that blockade of Panx1 channels during UTP stimulation enhances

ATP release from osteocytes. The mechanism is difficult to explain but the following observations may provide an indication. Panx1 channels have been proposed to associate with both $P2Y_2$ and P2X7 receptor subtypes. Osteocytes express both $P2Y_2$ and P2X7 receptors (Fig. 1, Table 2) [28]. Panx1 currents have been observed to be potently inhibited by CBX and high concentrations of both ATP and UTP, whereas P2X7 receptor currents are observed to be increased 5–10 % by CBX (50 μM) [50]. UTP leads to ATP release (Fig. 4) possibly via $P2Y_2$ receptor activation and prolonged ATP stimulation of P2X7 receptors leads to pore-opening and release of large quantities of ATP. Panx1 has been suggested as the P2X7 receptor-associated pore, but the P2X7 receptor channel itself can also be dilated to mediate the passage of larger molecules such as fluorescent dyes [52, 53]. Thus, if we assume that Panx1 channels are inhibited by CBX and ^{10}panx1 in our experiments, enhanced UTP induced ATP release via $P2Y_2$ receptor activation could be due to release via P2X7 receptors or a Panx1 cross talk effect on the P2X7 receptor channel. Alternatively, the enhanced ATP release could be a compensatory response to blocked Panx1 channels by the up-regulation of, e.g. exocytotic ATP release. However, as the blockers used are "dirty" and thus inhibits not only the channels intended but also a range of other channels, more specific inhibition of channels with either siRNA or the use of osteocytes from mice with targeted deletion of the individual channels should be done in order to more specifically address the mechanism for the increased release of ATP.

In conclusion, previous and present data indicate that osteocytes express P2X2, P2X7, $P2Y_2$ and/or $P2Y_4$ and $P2Y_{12}$ and/or $P2Y_{13}$ receptors, and since they all lead to intracellular calcium signalling upon stimulation, they are possible candidates in osteocyte intercellular signalling. Present data further indicate that, in addition to mechanical stimulation, osteocytes are able to release ATP in response to two other stimuli, cell rupture and nucleotide stimulation. Moreover, they can translate UTP stimulation into ATP release via $P2Y_2$ and/or $P2Y_4$ receptor activation. UTP-induced ATP release was found to be mediated by vesicular exocytosis and was graded according to the initial level of nucleotide stimulation, indicating that osteocytes can transduce fine-tuned nucleotide signals in the network. Since previous data further demonstrated that mechanical sensitivity of osteocytes is linked to acute ATP release, we hypothesise that P2 receptor-mediated nucleotide signalling is a central pathway in osteocyte mechanotransduction.

Based on previous and present data, we propose the following model for P2 receptor-mediated nucleotide signalling in osteocytes: (a) In mechanotransduction, the mechanical stimulation such as intra-lacunocanalicular fluid flow activates mechanosensors on the osteocyte surface, leading to load-dependent release of nucleotides. (b) Microfractures involving osteocytic cell rupture releases large amounts of ATP locally within the bone. Under both circumstances, the released nucleotides may be pushed through the lacunocanalicular network by pressure gradients and associated fluid flow and may activate P2 receptors expressed on neighbouring osteocytes. P2 receptor activation results in intracellular calcium signalling and dose-dependent vesicular ATP release, which serves to propagate the nucleotide signal through the bone. Eventually, this cascade of P2 receptor-mediated nucleotide signalling may reach the osteoblasts and osteoclasts on the bone surface, potentially leading to bone remodelling. These findings support the very intriguing hypotheses put forward independently by the groups of Timothy Skerry [54] and Charles H. Turner [55] that osteocytes may resemble a "neural network" in bone as excitatory agents usually found in the central nervous system also have roles in osteogenesis coupled to mechanical loading. We hypothesize that this network mediates nucleotide messages from the interior of bone telling that mechanical stimulation or structural damage has occurred and the magnitude of it.

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