P2 receptor networks regulate signaling duration over a wide dynamic range of ATP concentrations

Matthew W. Grol1, Alexey Pereverzev2, Stephen M. Sims2 and S. Jeffrey Dixon2,*

1Department of Anatomy and Cell Biology, Schulich School of Medicine and Dentistry, London, ON N6A 5C1, Canada
2Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, London, ON N6A 5C1, Canada

*Author for correspondence (jeff.dixon@schulich.uwo.ca)

Accepted 12 May 2013
Journal of Cell Science 126, 3615–3626
© 2013, Published by The Company of Biologists Ltd
doi: 10.1242/jcs.122705

Summary

The primordial intercellular signaling molecule ATP acts through two families of cell-surface P2 receptors – the P2Y family of G-protein-coupled receptors and the P2X family of ligand-gated cation channels. Multiple P2 receptors are expressed in a variety of cell types. However, the significance of these networks of receptors in any biological system remains unknown. Using osteoblasts as a model system, we found that a low concentration of ATP (10 μM, ATPlow) induced transient elevation of cytosolic Ca2+, whereas a high concentration of ATP (1 mM, ATPhigh) elicited more sustained elevation. Moreover, graded increases in the Ca2+ signal were achieved over a remarkable million-fold range of ATP concentrations (1 nM to 1 mM). Next, we demonstrated that ATPlow caused transient nuclear localization of the Ca2+-regulated transcription factor NFATc1; whereas, ATPhigh elicited more sustained localization. When stimulated with ATPhigh, osteoblasts from P2X7 loss-of-function mice showed only transient Ca2+-NFATc1 signaling; in contrast, sustained signaling was observed in wild-type cells. Additional experiments revealed a role for P2Y receptors in mediating transient signaling induced by low ATP concentrations. Thus, distinct P2 receptors with varying affinities for ATP account for this wide range of sensitivity to extracellular nucleotides. Finally, ATPhigh, but not ATPlow, was shown to elicit robust expression of the NFAT target gene Ptgs2 (encoding COX-2), consistent with a crucial role for the duration of Ca2+-NFAT signaling in regulating target gene expression. Taken together, ensembles of P2 receptors provide a mechanism by which cells sense ATP over a wide concentration range and transduce this input into distinct cellular signals.

Key words: ATP, Cytosolic calcium, Dose-duration coupling, NFATc1, P2X7, Purinergic

Introduction

Purine and pyrimidine nucleotides represent a primordial and pervasive class of intercellular chemical messengers (Burnstock and Verkhratsky, 2009). Many stimuli, including shear stress, mechanical stretch, osmotic swelling and hypoxia, trigger release of nucleotides from cells via mechanisms such as vesicular exocytosis and plasma membrane channels or transporters (Burnstock, 2007b). Once in the extracellular milieu, nucleotides act on target cells through two families of P2 nucleotide receptors – the P2Y family of G-protein-coupled receptors and the P2X family of ligand-gated cation channels (Abbracchio et al., 2006; Khakh and North, 2006).

Eight P2Y subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11–14) and seven subtypes of P2X (P2X1–7) have been identified in mammals, and are involved in regulating processes such as secretion, cell proliferation, differentiation, motility and death (Khakh and North, 2006; Burnstock, 2007a; Burnstock and Verkhratsky, 2010). Many P2Y receptors, including P2Y1, P2Y2, P2Y4 and P2Y6, couple to activation of phospholipase C (PLC), resulting in formation of inositol (1,4,5)-trisphosphate and subsequent release of Ca2+ from intracellular stores (Burnstock, 2007a). In contrast, homo- or heteromultimers of three ionotropic P2X receptor subunits form functional channels permeable to Na+, K+ and Ca2+ (Brown et al., 2010). Consequently, activation of P2X receptors results in membrane depolarization and, in many cases, Ca2+ influx. P2Y receptors are activated by one or more of ATP, ADP, UTP, UDP or UDP-glucose, with P2Y1, P2Y2, P2Y4, and P2Y11–13 exhibiting some degree of sensitivity for ATP; in contrast, members of the P2X receptor family are activated solely by ATP (Burnstock, 2007a).

All mammalian cell types express multiple P2 receptor subtypes, each with varying affinities for purine and pyrimidine nucleotides (Volonté et al., 2006; Burnstock and Verkhratsky, 2009). Bone-forming osteoblasts can express P2Y1, P2Y2, P2Y6, P2Y12–14 and P2X1–7, and several have been shown to regulate distinct processes in these cells (Orriss et al., 2010). For instance, activation of P2Y1 or P2Y2 enhances osteoblast responses to systemic factors such as parathyroid hormone (Bowler et al., 1999; Bowler et al., 2001). ATP or UTP, acting through P2Y2, inhibit matrix mineralization in cultures of differentiating osteoblasts (Hoebertz et al., 2002; Orriss et al., 2007). In contrast, activated P2X7 receptors enhance osteoblast differentiation and increase bone formation (Ke et al., 2003; Panupinthu et al., 2008). More recently, P2Y12 and P2Y13 have also been shown to positively regulate osteoblast proliferation and differentiation in vitro and in vivo (Syberg et al., 2012; Wang et al., 2012). Though the functions of individual P2Y and P2X receptors have been examined in osteoblasts and other cell types, mechanisms by which multiple P2 receptor subtypes act in concert to regulate cellular differentiation and function remain unclear in any system (Volonté et al., 2006).

The nuclear factor of activated T-cells (NFAT) family of transcription factors includes four members that are regulated by
cytosolic Ca$^{2+}$ (NFATc1–4) (Crabtree and Olson, 2002). In resting cells, NFATc1–4 are phosphorylated and localized to the cytosol. Activation-mediated elevation in the concentration of cytosolic free Ca$^{2+}$ ([Ca$^{2+}]_{i}$) leads to activation of calcineurin, a serine/threonine phosphatase that dephosphorylates NFATc1–4, resulting in their nuclear translocation and transcriptional activation (Hogan et al., 2003). Though classically described as master regulators of T-cell development and function, NFATc1–4 also play essential roles in the differentiation and function of neuronal, muscle and bone cells, including osteoblasts and osteoclasts (Hogan et al., 2003; Sitara and Aliprantis, 2010; Moore and Goldberg, 2011). However, whether P2 receptors signal through NFAT in bone-forming osteoblasts remains unexplored.

In the present study, we show that endogenous co-expression of multiple P2Y and P2X receptors provides a novel mechanism for dose-to-duration encoding of Ca$^{2+}$-NFAT signaling. Using osteoblasts as a model system, we found that increases in Ca$^{2+}$ signaling could be achieved over a million-fold range of ATP concentrations. Low concentrations of ATP acting through P2Y receptors caused transient elevation of Ca$^{2+}$ and brief nuclear localization of NFATc1, but failed to induce expression of NFAT target genes. In contrast, high ATP concentrations acting through P2X7 elicited sustained Ca$^{2+}$-NFATc1 signaling and robust NFAT transcriptional activity. Taken together, these data show that P2 receptor networks provide a mechanism by which cells sense ATP over a wide range of concentrations and transduce this input into distinct cellular signals.

Results

Effect of ATP concentration on the duration of cytosolic Ca$^{2+}$ signals

Cells of the osteoblast lineage express multiple subtypes of P2Y and P2X nucleotide receptors (Orriss et al., 2012). Given the central role for Ca$^{2+}$ in P2 receptor signaling, we investigated the functional significance of endogenous P2 receptor networks by assessing Ca$^{2+}$ signaling elicited by extracellular ATP. MC3T3-E1 osteoblast-like cells were loaded with the Ca$^{2+}$-sensitive dye fluo-4, and changes in [Ca$^{2+}]_{i}$ were monitored using real-time imaging of live-cells by confocal microscopy (Fig. 1A). A low concentration of ATP (10 µM, which activates some P2Y and all P2X receptor subtypes except P2X7; ATP$_{low}$) induced large, transient elevations in [Ca$^{2+}]_{i}$, that recovered to baseline within ~240 s after stimulation (data are whole-field Ca$^{2+}$ responses, Fig. 1A,B; supplementary material Movie 1). In contrast, a higher ATP concentration (1 mM, which activates some P2Y and all P2X receptor subtypes including P2X7; ATP$_{high}$) elicited larger, more sustained elevations in [Ca$^{2+}]_{i}$, exceeding 20 min in duration (Fig. 1A,B; supplementary material Movie 2). Vehicle elicited a small elevation in [Ca$^{2+}]_{i}$, consistent with fluid shear-induced release of ATP and slight P2 receptor activation (supplementary material Figs S1, S2), as described previously (Li et al., 2005).

To further characterize ATP-induced Ca$^{2+}$ signaling, changes in [Ca$^{2+}]_{i}$ were assessed in response to ATP concentrations from 1 nM to 10 mM. We first plotted the peak amplitude of the Ca$^{2+}$ signal against ATP concentration (Fig. 1C). Remarkably, graded increases in the peak Ca$^{2+}$ response were observed over a million-fold range of ATP concentrations (1 nM to 1 mM). The amplitude at 10 mM ATP was slightly reduced, likely reflecting chelation of extracellular Ca$^{2+}$ by this high concentration of ATP.

In contrast, only ATP concentrations >100 µM elicited a sustained Ca$^{2+}$ signal (measured as amplitude 10 min post-treatment, Fig. 1D). Similar patterns of concentration dependence were observed when percentages of cells exhibiting elevation of [Ca$^{2+}]_{i}$ were examined (supplementary material Fig. S3).

Finally, we quantified the magnitude of responses as the area under the fluorescence-time curve, providing a combined measure of both amplitude and duration. In this case, concentration dependence was clearly biphasic (Fig. 1E). The first phase began at ~1 nM and plateaued at ~1 µM ATP, whereas the second phase began at ~100 µM and plateaued at ~1 mM ATP.

P2X7 is essential for sustained cytosolic Ca$^{2+}$ signaling elicited by high concentrations of ATP

Extracellular ATP activates a number of P2Y and P2X receptor subtypes at concentrations within the nM to µM range; in contrast, P2X7 is stimulated solely at ATP concentrations exceeding 100 µM (Khakh and North, 2006; Burnstock, 2007a). Therefore, we investigated whether P2X7 receptors mediate the effects of high ATP concentrations. Changes in [Ca$^{2+}]_{i}$, were examined following treatment of osteoblast-like cells with vehicle, ATP$_{low}$ or BzATP (300 µM) in the absence or presence of A 438079 (10 µM), a specific P2X7 antagonist (Nelson et al., 2006; Donnelly-Roberts and Jarvis, 2007) (Fig. 2A–C). Although not specific for P2X7, BzATP is a more potent agonist than ATP at the P2X7 receptor (North, 2002). In the absence of antagonist, BzATP elicited a large, sustained elevation in cytosolic Ca$^{2+}$, whereas ATP$_{low}$ induced a more transient increase (Fig. 2A–C). A 438079 abolished the sustained phase of the BzATP-induced response, converting it to a transient increase comparable to that elicited by ATP$_{low}$ (Fig. 2A–C). A 438079 had no effect on Ca$^{2+}$ elevation induced by ATP$_{low}$, consistent with the specificity of this P2X7 receptor antagonist.

To confirm the role of P2X7 in mediating effects of high concentrations of ATP or BzATP on cytosolic Ca$^{2+}$ signaling, changes in [Ca$^{2+}]_{i}$, were examined in primary calvarial osteoblasts isolated from wild-type mice and mice with loss of P2X7 function (knockout) (Fig. 2D–F). Stimulation of wild-type cells with vehicle, ATP$_{low}$ or BzATP (300 µM) both elicited more sustained elevations of [Ca$^{2+}]_{i}$, exceeding 20 min in duration. In contrast, responses to ATP$_{high}$ in calvarial osteoblasts from knockout mice were comparable to those induced by ATP$_{low}$, and treatment with BzATP had no significant effect on [Ca$^{2+}]_{i}$ (Fig. 2D–F). Taken together, pharmacological and genetic evidence establish that the sustained elevations in [Ca$^{2+}]_{i}$, induced by BzATP or high concentrations of ATP are mediated by activation of P2X7 receptors.

Source of Ca$^{2+}$ underlying the transient and sustained elevations of [Ca$^{2+}]_{i}$, elicited by ATP

Several P2Y receptors couple to activation of PLC, resulting in release of Ca$^{2+}$ from intracellular stores (Burnstock, 2007a). In contrast, P2X receptors form channels that in many cases permit Ca$^{2+}$ influx (Browne et al., 2010). We next determined the source of Ca$^{2+}$ required for the transient and sustained elevations of [Ca$^{2+}]_{i}$, To assess the contribution of intracellular stores, changes in [Ca$^{2+}]_{i}$, were examined following treatment with vehicle, ATP$_{low}$ or BzATP (300 µM) in the absence or presence of the
PLC inhibitor U 73122 (1 µM) (Fig. 3A–C). U 73122 abolished the response induced by ATP<sub>low</sub> and the transient component of the Ca<sup>2+</sup> signal elicited by BzATP. In contrast, the inhibitor had no significant effect on the sustained phase of the BzATP-induced response (Fig. 3A–C).

To determine the role of extracellular Ca<sup>2+</sup>, changes in [Ca<sup>2+</sup>]<sub>i</sub> were examined following treatment of cells with vehicle, ATP<sub>low</sub> or BzATP (300 µM) in the presence or absence of extracellular Ca<sup>2+</sup> (Fig. 3D–F). Removal of extracellular Ca<sup>2+</sup> abolished the sustained phase of the BzATP-induced response. In contrast, there was no significant effect on the transient component of the responses to ATP<sub>low</sub> and BzATP (Fig. 3D–F). Taken together, these data are consistent with ATP<sub>low</sub> activating P2Y receptors, leading to release of Ca<sup>2+</sup> from intracellular stores. On the other hand, BzATP activates both P2Y receptors and P2X7, leading to transient release of Ca<sup>2+</sup> from intracellular stores and sustained influx, respectively.

We further investigated the nature of the P2Y receptors mediating transient Ca<sup>2+</sup> signaling. UTP (10 µM) elicited responses comparable to those elicited by ATP<sub>low</sub>, providing further evidence for involvement of P2Y receptors. Next, we examined responses to sequential addition of ATP<sub>low</sub> and UTP and observed marked cross-desensitization (supplementary material Fig. S4), consistent with the agonist specificities of P2Y<sub>2</sub> and/or P2Y<sub>4</sub> (Burnstock, 2007a).

**Effect of ATP concentration on the duration of NFATc1 nuclear localization**

Elevations in [Ca<sup>2+</sup>]<sub>i</sub> can activate calcineurin, resulting in NFATc1 translocation from the cytoplasm to the nucleus (Crabtree and Olson, 2002; Hogan et al., 2003). To characterize the dynamics of NFATc1 activation in response to ATP, osteoblast-like cells were transfected with EGFP-tagged NFATc1, and subcellular localization was monitored by confocal imaging of live cells. In the absence of agonists, NFATc1–EGFP was uniformly distributed throughout the cytoplasm, with little if any fluorescence in the nucleus (supplementary material Fig. S5A). ATP (0.01–1 mM) and BzATP (300 µM) all induced prompt translocation of NFATc1–EGFP to the nucleus. Low

**Fig. 1. Elevations in cytosolic free Ca<sup>2+</sup> induced by P2 nucleotide receptors are dependent on ATP concentration.** MC3T3-E1 cells were loaded with the Ca<sup>2+</sup>-sensitive dye fluo-4 and changes in [Ca<sup>2+</sup>]<sub>i</sub> were monitored by confocal microscopy. Concentrations of ATP indicated are final concentrations in the bath. Each concentration was tested on separate cell samples. (A) Representative fields of cells treated with vehicle, ATP<sub>low</sub> (10 µM) or ATP<sub>high</sub> (1 mM). Scale bar: 20 µm. Supplementary material Movies 1 and 2 show responses to ATP<sub>low</sub> and ATP<sub>high</sub>, respectively. (B) Changes in [Ca<sup>2+</sup>]<sub>i</sub> were quantified from the average responses of cells in a single field as (F/F<sub>o</sub>)-1, where F is fluorescence intensity and F<sub>o</sub> is baseline fluorescence observed prior to treatment. At the point indicated by the arrows, cells were treated with vehicle (Veh), ATP<sub>low</sub> or ATP<sub>high</sub>. Traces are representative of responses from eight independent preparations. (C) Peak amplitude was quantified as the maximal rise in [Ca<sup>2+</sup>]<sub>i</sub>, above basal levels. Responses to vehicle are indicated by the letter V. (D) The sustained phase was quantified as the amplitude of the Ca<sup>2+</sup> response at 10 min post-treatment. (E) Change in [Ca<sup>2+</sup>]<sub>i</sub> was quantified as the area under the curve, providing a combined measure of amplitude and duration. The area was determined from the beginning of agonist-induced elevation in [Ca<sup>2+</sup>]<sub>i</sub> until cytosolic Ca<sup>2+</sup> recovered to within 15% of baseline. The inset shows an expanded view. Data in C–E are means±s.e.m. (n=8 independent preparations).
concentrations of ATP (10–100 μM) elicited transient nuclear localization of NFATc1–EGFP, with recovery of cytosolic fluorescence less than 90 min after stimulation (supplementary material Fig. S5 and Movie 3). In contrast, ATP\textsubscript{high} and BzATP both induced more sustained nuclear localization of NFATc1–EGFP that persisted for at least 2 h (supplementary material Fig. S5 and Movie 4).

To quantify NFATc1 activation, osteoblast-like cells expressing NFATc1–EGFP were treated and fixed at various time points. The percentage of cells exhibiting nuclear localization of NFATc1–EGFP was determined and expressed as a function of nucleotide concentration or time. When assessed 15 min after addition of nucleotide, nuclear localization of NFATc1–EGFP was induced in a concentration-dependent manner over a relatively narrow range for ATP and UTP (from \(\sim 0.1\) to 1 μM) and BzATP (from \(\sim 1\) to 100 μM) (Fig. 4A). Responses to low concentrations of ATP and UTP and higher concentrations of BzATP indicate involvement of multiple P2 receptor subtypes. Moreover, at this early time point, NFATc1 responds to ATP over a relatively narrow dynamic range of concentrations.

We next examined the time course of NFATc1 nuclear translocation. Consistent with the live-cell data (supplementary material Fig. S5), nuclear localization of NFATc1–EGFP elicited by ATP\textsubscript{low} was transient, with recovery of cytosolic localization in all cells by 90 min (Fig. 4B,C). In contrast, ATP\textsubscript{high} and BzATP both induced sustained localization of NFATc1–EGFP to the nucleus that persisted for up to 3 h (Fig. 4B,C). Thus, elevations in \([\text{Ca}^{2+}]_i\) elicited by increasing ATP concentrations are transduced into graded increases in the duration of NFATc1 nuclear localization. Moreover, effects of BzATP and ATP\textsubscript{high} implicate the P2X7 receptor in mediating prolonged NFATc1 nuclear localization.

**P2X7 is essential for sustained NFATc1 nuclear localization elicited by high concentrations of ATP**

To investigate whether P2X7 receptors mediate sustained NFATc1 nuclear localization, MC3T3-E1 cells were transfected with EGFP-tagged NFATc1 and treated with vehicle, ATP\textsubscript{low} or BzATP (300 μM) in the absence or presence of A 438079 (10 μM). A 438079 abolished the sustained phase of the BzATP-induced response, converting it to a transient response comparable to that elicited by ATP\textsubscript{low} (Fig. 5A,B). In contrast, this antagonist had no effect on the transient nuclear localization of NFATc1–EGFP stimulated by ATP\textsubscript{low}.

**Fig. 2. The P2X7 receptor is required for sustained elevations in \([\text{Ca}^{2+}]_i\).**

(A) \([\text{Ca}^{2+}]_i\) of MC3T3-E1 cells loaded with fluo-4 was monitored using confocal microscopy. Cells were incubated for 5–10 min in the absence or presence of the specific P2X7 antagonist A 438079 (10 μM). At the point indicated by the arrows, cells were then treated with vehicle (Veh), ATP\textsubscript{low} (10 μM) or BzATP (300 μM) in the absence or presence of A 438079 (10 μM). A 438079 abolished the sustained phase of the BzATP-induced response, converting it to a transient response comparable to that elicited by ATP\textsubscript{low} (Fig. 5A,B). In contrast, this antagonist had no effect on the transient nuclear localization of NFATc1–EGFP stimulated by ATP\textsubscript{low}.
To confirm the role of P2X7 receptors in mediating sustained nuclear localization of NFATc1, primary calvarial osteoblasts were isolated from P2X7 knockout and wild-type mice. Cultures were treated with vehicle, ATP\textsubscript{low}, ATP\textsubscript{high} or BzATP (300 μM), and fixed at 15 or 120 min (Fig. 5C,D). Endogenous NFATc1 was labeled using a monoclonal antibody and localized by immunofluorescence (Fig. 5C). The percentage of cells exhibiting nuclear localization was determined for each treatment at 15 and 120 min (Fig. 5D). Stimulation of wild-type calvarial osteoblasts with ATP\textsubscript{low} induced transient NFATc1 nuclear localization, whereas ATP\textsubscript{high} and BzATP both elicited sustained localization (Fig. 5C,D). In contrast, responses of osteoblasts from knockout mice to ATP\textsubscript{high} and BzATP were transient and comparable to those elicited by ATP\textsubscript{low} (Fig. 5C,D). Approximately 35% of wild-type osteoblasts responded to ATP\textsubscript{high} and BzATP at 120 min (Fig. 5D, right panel), in keeping with the percentage of primary calvarial osteoblasts reported previously to express functional P2X7 receptors (Panupinthu et al., 2007).

Taken together, these experiments establish that the sustained nuclear localization of NFATc1 elicited by BzATP or high concentrations of ATP is mediated by activation of P2X7 receptors; whereas, transient localization induced by low concentrations of ATP is due to activation of higher affinity P2 receptors.

Effects of nucleotides on NFAT transcriptional activity
To determine if differences in duration of P2 receptor-induced Ca\textsuperscript{2+}-NFATc1 signaling give rise to corresponding alterations in expression of NFAT target genes, MC3T3-E1 cells were treated with vehicle, ATP\textsubscript{low}, ATP\textsubscript{high} or BzATP (300 μM). Total RNA was isolated at various times, and expression of the NFATc1 target gene \textit{Ptgs2} (encoding COX-2) was assessed by real-time RT-PCR (Fig. 6A). BzATP and ATP\textsubscript{high} elicited dramatic increases in expression of \textit{Ptgs2} that peaked at 3 h before returning to baseline by 6 h after treatment. In contrast, ATP\textsubscript{low} did not induce any increase in \textit{Ptgs2} expression.

The \textit{Ptgs2} promoter contains binding sites for many transcription factors in addition to NFAT (Kang et al., 2007). To more specifically assess changes in NFAT transcriptional activity, osteoblast-like cells were transfected with an NFAT luciferase reporter and treated with vehicle, ATP\textsubscript{low}, ATP\textsubscript{high} or BzATP (300 μM). After 24 h, cell lysates were collected and luminescence was assessed as a measure of NFAT transcriptional activity (Fig. 6B). Similar to the pattern observed for \textit{Ptgs2}, BzATP and ATP\textsubscript{high} both elicited significant increases in NFAT transcriptional activity, whereas ATP\textsubscript{low} had no effect.

Fig. 3. Distinct sources of Ca\textsuperscript{2+} underlie transient and sustained elevations in [Ca\textsuperscript{2+}]. MC3T3-E1 cells were loaded with the Ca\textsuperscript{2+}-sensitive dye indo-1 and suspended in HEPES buffer in a fluorometric cuvette with continuous stirring. Changes in [Ca\textsuperscript{2+}], were monitored by fluorescence spectrophotometry, and quantified as the ratio of emission intensity at 405 nm to that at 485 nm. (A) Cells were incubated for 300 s in the absence or presence of the PLC inhibitor U 73122 (1 μM) in Ca\textsuperscript{2+}-containing HEPES buffer. At the point indicated by the arrows, cells were then treated with vehicle (Veh), ATP\textsubscript{low} (10 μM) or BzATP (300 μM). Traces are representative of responses from four independent preparations. (B,E) Ca\textsuperscript{2+} elevations were analyzed for their (B) peak amplitude and (E) sustained phase. α indicates significant difference from vehicle; β indicates significant difference between ATP\textsubscript{low} and BzATP (P<0.05). Data are means±s.e.m. (n=7 samples from four independent preparations). (D) Cells were incubated for 100 s in the absence or presence of the PLC inhibitor U 73122 (1 μM) in Ca\textsuperscript{2+}-containing or nominally Ca\textsuperscript{2+}-free HEPES buffer, respectively. At the point indicated by the arrows, cells were then treated with vehicle (Veh), ATP\textsubscript{low} (10 μM) or BzATP (300 μM). Traces are representative of responses from four independent preparations. (C,F) Ca\textsuperscript{2+} elevations were analyzed for amplitudes of the peak (C) and sustained phase (F). α indicates significant difference from vehicle; β indicates significant difference between ATP\textsubscript{low} and BzATP (P<0.05). Data are means±s.e.m. (n=6 samples from four independent preparations).
transcriptional activity. In contrast, ATP.low did not induce any significant change compared to vehicle (Fig. 6B). Taken together, sustained Ca\(^{2+}\)-NFATc1 signaling elicited by high concentrations of ATP or BzATP is associated with robust expression of NFAT target genes.

To confirm a role for the Ca\(^{2+}\)-calcineurin pathway in regulation of NFAT transcriptional activity, changes in Ptgs2 expression and NFAT reporter activity were examined (supplementary material Fig. S6). Osteoblast-like cells were treated with vehicle, ATP.low, ATP.high or BzATP (300 \(\mu\)M) in the absence or presence of the calcineurin inhibitors cyclosporin A (1 \(\mu\)M) or FK506 (1 \(\mu\)M). Both cyclosporin A and FK506 significantly suppressed expression of Ptgs2 induced by ATP.high and BzATP (supplementary material Fig. S6A). Moreover, in osteoblast-like cells transfected with the NFAT luciferase reporter, the effects of BzATP and ATP.high were abolished by inhibition of calcineurin (supplementary material Fig. S6B). These observations are consistent with nucleotide-induced changes in Ptgs2 expression and NFAT reporter activity being mediated by activation of the Ca\(^{2+}\)-calcineurin pathway.

**P2X7 is essential for mediating effects of high concentrations of ATP on NFAT transcriptional activity**

Changes in Ptgs2 expression and NFAT reporter activity were examined in osteoblast-like cells following treatment with vehicle, ATP.low, ATP.high or BzATP (300 \(\mu\)M) in the absence or presence of A 438079 (10 \(\mu\)M). A 438079 completely abolished the effects of ATP.high and BzATP both on expression of Ptgs2 (Fig. 7A) and on luminescence in osteoblast-like cells transfected with the NFAT luciferase reporter (Fig. 7B).

To confirm the involvement of P2X7 receptors, changes in Ptgs2 expression and NFAT reporter activity were examined in primary calvarial osteoblasts isolated from P2X7 knockout and wild-type mice. Stimulation of wild-type calvarial osteoblasts with ATP.high or BzATP (300 \(\mu\)M) induced significant expression of Ptgs2 at 3 h, whereas ATP.low had no effect (Fig. 7C). In contrast, ATP.low, ATP.high and BzATP all failed to elicit expression of Ptgs2 in calvarial osteoblasts from knockout mice (Fig. 7C). Moreover, in primary calvarial osteoblasts transfected with the NFAT luciferase reporter, ATP.high induced an increase in luminescence that was absent in cells from knockout mice (Fig. 7D). These observations establish that the increase in NFAT transcriptional activity and target gene expression elicited by BzATP or high concentrations of ATP is mediated by activation of the P2X7 receptor.

**Discussion**

In this study, we examined ATP-induced Ca\(^{2+}\)-NFAT signaling through a network of endogenously expressed P2 nucleotide receptors. We show that ensembles of P2Y and P2X receptor subtypes impart sensitivity over a wide range of ATP concentrations, and provide a mechanism by which cells transduce ATP levels into distinct cellular signals (Fig. 8). Specifically, low concentrations of ATP act through P2Y receptors to elicit transient Ca\(^{2+}\)-NFAT signaling; whereas, high ATP concentrations act through P2X7 to induce more sustained Ca\(^{2+}\)-NFAT signaling that culminates in robust target gene expression.

**Fig. 4. Duration of NFATc1 nuclear localization is dependent on ATP concentration.** MC3T3-E1 cells were transfected with plasmids encoding EGFP-tagged NFATc1. (A) Cells were treated with the indicated concentrations of UTP, ATP or BzATP and fixed at 15 min. The number of cells exhibiting nuclear localization was expressed as a percentage of the total number of transfected cells. Data are means±s.e.m. (\(n=3\) independent experiments performed in triplicate). (B) Cells were treated with vehicle, ATP.low (10 \(\mu\)M), ATP.high (1 mM) or BzATP (300 \(\mu\)M) and fixed at the indicated times. Nuclei were stained with DAPI (blue). Images are representative of responses from four independent preparations. Scale bar: 20 \(\mu\)m. (C) Cells were treated with vehicle, ATP.low, ATP.high or BzATP, and fixed at time points from 0 to 240 min. The percentage of cells exhibiting nuclear localization of NFATc1 was quantified. Data are means±s.e.m. (\(n=4\) independent experiments performed in triplicate).
expression. These findings demonstrate that the Ca\textsuperscript{2+}-NFAT pathway functions in dose-to-duration encoding of P2 receptor stimuli.

**P2 receptor networks impart sensitivity over a wide dynamic range of ATP concentrations**

Previous studies examining the characteristics of individual P2Y and P2X receptor subtypes have demonstrated that each exhibits distinct affinities for purine and pyrimidine nucleotides. Of the P2 receptor subtypes sensitive to ATP, P2Y\textsubscript{2}, P2X1, P2X3 and P2X5 have the highest affinities, with responses seen at relatively low ATP concentrations (<1 \mu M); in contrast, P2X7 has the lowest affinity with activation occurring at much higher concentrations of ATP (Ralevic and Burnstock, 1998). Though informative, many of these earlier studies used heterologous expression to characterize individual P2 receptor subtypes. However, expression of a single P2Y or P2X subtype does not recapitulate responses mediated by a network of P2 receptors. Moreover, overexpression of a single receptor could perturb interactions among endogenous P2 receptors, such as P2Y dimerization and the formation of heteromeric P2X receptors. Thus, results of these earlier studies may not represent the physiological behavior of P2 receptor networks. For these reasons, we examined concentration-dependent responses to ATP mediated by a network of P2 receptors endogenously expressed in osteoblasts.

Characterization of the concentration dependence of ATP-induced Ca\textsuperscript{2+} signaling in osteoblasts revealed, for the first time in any system, that graded increases in the Ca\textsuperscript{2+} response could be achieved over a remarkably broad range of ATP concentrations (1 nM to 1 mM). The physiological effects of many other intercellular messengers are also mediated by multiple receptor subtypes. However, few if any of these receptor families exhibit as wide a range of affinities as those observed for ATP in the purinergic system. For instance, prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) acts through four G-protein-coupled receptors (EP\textsubscript{1–4}), which exhibit an ~100-fold range in EC\textsubscript{50} for PGE\textsubscript{2} (Abramovitz et al., 2000; Hata and Breyer, 2004). LPA, another lipid mediator, acts through at least five G-protein-coupled receptors, LPA\textsubscript{1–5}, with a similar 100-fold range in EC\textsubscript{50} (Bandoh et al., 2000; Anliker and Chun, 2004; Lee et al., 2006). On the other hand, the excitatory neurotransmitter L-glutamate, which like ATP signals

---

**Fig. 5.** The P2X7 receptor is required for sustained NFATc1 nuclear localization. (A) MC3T3-E1 cells were transfected with plasmids encoding EGFP-tagged NFATc1. Cells were incubated for 5–10 min in the absence or presence of A 438079 (10 \mu M). Next, cells were treated with vehicle, ATP\textsubscript{low} (10 \mu M) or BzATP (300 \mu M) and fixed at the indicated times. Images are representative responses of cells from six independent preparations. Scale bar: 20 \mu m. (B) The percentage of MC3T3-E1 cells exhibiting NFATc1 nuclear localization was quantified. \(\alpha\) indicates significant difference between BzATP and ATP\textsubscript{low} (\(P<0.05\)). Data are means±s.e.m. (\(n=6\) independent experiments performed in triplicate). (C) Calvarial osteoblasts from wild-type (WT) and P2X7-knockout (KO) mice were treated with vehicle, ATP\textsubscript{low} (10 \mu M), ATP\textsubscript{high} (1 mM) or BzATP (300 \mu M) and fixed at 15 or 120 min. Subcellular localization of endogenous NFATc1 was detected by immunofluorescence (green). Nuclei were stained with DAPI (blue). Images are representative responses from three or four separate preparations. Scale bar: 20 \mu m. (D) The percentage of calvarial osteoblasts exhibiting NFATc1 nuclear localization was quantified. \(\alpha\) indicates significant difference from vehicle within the same genotype; \(\beta\) indicates significant difference between ATP\textsubscript{high} or BzATP and ATP\textsubscript{low} within the same genotype (\(P<0.05\)). Note the expanded y-axis scale in right panel. Data are means±s.e.m. (\(n=6–8\) samples from 3–4 independent preparations).
through families of metabotropic and ionotropic receptors, elicits responses at low and high concentrations of L-glutamate (Conn and Pin, 1997; Traynelis et al., 2010). However, it has not been reported that L-glutamate can induce graded, dose-dependent increases in the amplitude or duration of a common intracellular signal. Nevertheless, given its similarities to the purinergic system, the principles described in the present study for ATP signaling may also be applicable to the glutamnergic system.

**P2 receptor networks enable dose-to-duration encoding of Ca\(^{2+}\)-NFAT signaling**

In addition to relaying qualitative information about the presence or absence of a stimulus, receptors and their associated signaling pathways must also transmit quantitative information about stimulus intensity. ‘Dose-to-duration’ encoding refers to the process by which information about the concentration of a stimulus is transduced as duration of the signal, permitting distinct cellular responses to different agonist concentrations (Behar et al., 2008). In yeast, the switch from proliferation to differentiation and/or mating is dependent both on pheromone concentration and the duration of downstream mitogen-activated protein kinase (MAPK) activity. Behar et al. proposed a model in which negative feedback regulation of the yeast pheromone receptor causes dose-dependent decreases in its affinity for ligand to allow for signaling beyond initial saturation (Behar et al., 2008).

As opposed to regulating the affinity of a single receptor, we describe a novel mechanism for dose-to-duration encoding in which the presence of multiple P2Y and P2X receptors, with different affinities for ATP, determine the duration of Ca\(^{2+}\)-NFATc1 signaling. We demonstrated that low concentrations of ATP (1 nM to 100 μM) induce transient Ca\(^{2+}\) elevation and NFATc1 activation, whereas higher ATP concentrations (300 μM to 10 mM) elicit more sustained Ca\(^{2+}\)-NFATc1 signaling.

Ca\(^{2+}\) signaling is a central mechanism by which P2 receptors elicit changes in cell behavior in response to extracellular nucleotides. A number of groups have shown that ATP and other nucleotides can elicit transient or sustained Ca\(^{2+}\) signals in a variety of other cell types (Oshimi et al., 1999; Möller et al., 2000; Nobile et al., 2003; Korcok et al., 2004). The role of the Ca\(^{2+}\)-regulated NFAT transcription factors in P2 receptor signaling has also been examined previously (Ferrari et al., 1999; Abbott et al., 2000; Kataoka et al., 2009; Shiratori et al., 2010). However, no previous studies have employed a concentration range capable of revealing the relationship between ATP dose and Ca\(^{2+}\)-NFAT signal duration described in the present study. Moreover, we provide the first evidence demonstrating that P2 receptors utilize the duration of Ca\(^{2+}\)-NFAT signaling to elicit distinct responses to different concentrations of extracellular ATP.

In general, signal duration can control distinct cell functions in a variety of biological systems. In the neuronal PC12 cell line, transient activation of extracellular-signal-regulated kinase (ERK) by epidermal growth factor stimulates proliferation; whereas prolonged ERK activation, elicited by nerve growth factor, promotes differentiation (Vaudry et al., 2002). Similarly, in yeast, vegetative growth patterns are mediated by transient MAPK signaling at low pheromone levels; whereas, growth arrest and mating are initiated by sustained MAPK signaling in response to high levels of pheromone (Dohlian and Thorner, 2001). In the present study, we found that transient Ca\(^{2+}\)-NFAT signaling elicited by low concentrations of ATP resulted in no measurable increase in NFAT transcriptional activity. Nevertheless, these Ca\(^{2+}\) transients regulate other important aspects of osteoblast function. For example, such Ca\(^{2+}\) events activate ion channels, regulate cytoskeletal remodeling and stimulate secretion in other cell types (Berridge, 2012). In this regard, low concentrations of ATP have been reported to induce functional changes in cells of the osteoblast lineage. For instance, stimulation of P2 receptors with micromolar concentrations of ATP increases the proliferation of osteoblasts and osteoblast-like cells (Nakamura et al., 2000; Katz et al., 2011).

In addition, micromolar concentrations of ATP and UTP activate the osteoblast master transcription factor runt-related transcription factor 2 in the osteoblast-like HOBIT cell line (Costessi et al., 2005). In addition to these previously reported effects of low ATP concentrations, we found that high concentrations of ATP elicited sustained Ca\(^{2+}\)-NFATc1 signaling to stimulate NFAT transcriptional activity. Thus, we demonstrate that different ATP concentrations are transduced into distinct cellular signals.

**Potential physiological roles of P2 receptor networks in osteoblasts**

Mechanotransduction, the process by which mechanical stimuli are translated into cellular responses, has been suggested to be mediated by nucleotide release and subsequent P2 receptor signaling in bone (Dixon and Sims, 2000; Robling et al., 2006).
Phenotypic examination of knockout mouse strains for various P2Y and P2X receptor subtypes has indeed revealed a number of skeletal phenotypes, helping to solidify the importance of purinergic signaling in bone. Whole-body deletion of P2Y6, P2Y12 or P2Y13 results in decreased bone resorption, and loss of P2Y13 leads to decreased bone formation (Orriss et al., 2011a; Orriss et al., 2011b; Su et al., 2012; Wang et al., 2012). Furthermore, genetically modified mice carrying a non-functional P2X7 receptor exhibit diminished periosteal bone formation, excessive trabecular bone resorption, and impaired skeletal responses to mechanical loading (Ke et al., 2003; Li et al., 2005). The findings of the present study provide the first evidence that expression of multiple P2 receptor subtypes increases the range over which differences in ATP concentration can be sensed by a cell. This phenomenon provides a novel mechanism by which osteoblasts may transduce differences in ATP concentration and, therefore, intensity of mechanical stimuli over a remarkably wide dynamic range.

We also demonstrate for the first time that P2X7 receptors couple to the Ca\(^{2+}\)-NFATc1 pathway in osteoblasts. Our lab has previously demonstrated that activation of P2X7 receptors by exogenous nucleotides leads to production of LPA and PGE\(_2\) by osteoblasts, culminating in enhanced differentiation and matrix mineralization (Panupinthu et al., 2008). The Ca\(^{2+}\)-NFATc1 pathway also plays an important role in the regulation of osteoblast differentiation (Koga et al., 2005; Winslow et al., 2006), but until now the pathways underlying NFATc1 activation in osteoblasts have remained obscure. In the present study, we found that Ca\(^{2+}\)-NFATc1 signaling stimulates expression of COX-2 downstream of the P2X7 receptor in osteoblasts. Given the important role of COX-2 and PGE\(_2\) in osteoblast differentiation and responses to mechanical stimuli (Blackwell et al., 2010), we propose that the Ca\(^{2+}\)-NFATc1 axis may play an

Fig. 7. The P2X7 receptor is required for changes in NFAT transcriptional activity elicited by ATP\(_{\text{high}}\) or BzATP. (A) MC3T3-E1 cells were incubated for 5–10 min in the absence (Control) or presence of A 438079 (10 \(\mu\)M). Next, cells were treated for 3 h with vehicle, ATP\(_{\text{low}}\) (10 \(\mu\)M), ATP\(_{\text{high}}\) (1 mM) or BzATP (300 \(\mu\)M) in the continued absence or presence of antagonist. Real-time RT-PCR was performed to assess expression levels of \(\text{Ptgs2}\). Data were normalized to levels of 18S and are shown relative to values for control cultures treated with vehicle. Note the expanded y-axis scale. (B) MC3T3-E1 cells transfected with the NFAT luciferase reporter plasmid were incubated for 5–10 min in the absence or presence of A 438079 (10 \(\mu\)M). Next, cells were treated with vehicle, ATP\(_{\text{low}}\), ATP\(_{\text{high}}\) or BzATP in the continued absence or presence of antagonist. After 24 h, cell lysates were collected and luminescence was assessed. Luminescence was expressed relative to values for wild-type cultures treated with vehicle. For both A and B, \(\alpha\) indicates significant difference from vehicle; \(\beta\) indicates significant difference between ATP\(_{\text{low}}\) or BzATP and ATP\(_{\text{low}}\) \((P<0.05)\). Data are means±s.e.m. \((n=9\) samples from three independent preparations) . (C) Primary osteoblasts from wild-type and P2X7-knockout mice were treated with vehicle, ATP\(_{\text{low}}\), ATP\(_{\text{high}}\) or BzATP, and total RNA was isolated at 3 h. Real-time RT-PCR was performed to assess expression levels of \(\text{Ptgs2}\). Data were normalized to levels of 18S and are shown relative to values for wild-type cultures treated with vehicle. (D) Primary osteoblasts were transfected with an NFAT luciferase reporter plasmid and treated with vehicle, ATP\(_{\text{low}}\) or ATP\(_{\text{high}}\). After 24 h, cell lysates were collected and luminescence was assessed. Luminescence was expressed relative to values for wild-type cultures treated with vehicle. For both C and D, \(\alpha\) indicates significant difference from vehicle within the same genotype; \(\beta\) indicates significant difference between ATP\(_{\text{low}}\), ATP\(_{\text{high}}\), or BzATP and ATP\(_{\text{low}}\) within the same genotype \((P<0.05)\). Data are means±s.e.m. \((n=9–12\) samples from three or four independent preparations).
concentrations triggers sustained influx of Ca2+ and prolonged NFATc1 target genes (left). In contrast, activation of the P2X7 receptor by high ATP results in transient release of Ca2+. This transient elevation of [Ca2+]i causes brief NFATc1 nuclear localization, resulting in robust NFAT target gene expression (right). Low ATP concentrations leads to activation of phospholipase C (PLC) and distinct cellular signals. Stimulation of one or more P2Y receptor subtypes by ATP of extracellular fluid under different circumstances – smaller amounts of ATP are released into the extracellular space of cells (Gonzalez-Frutos et al., 2006). This can evoke transient activation of P2Y subtypes, resulting in Ca2+ signaling and NFATc1 nuclear localization, but fails to elicit expression of NFATc1 target genes (right). The ability of ATP to elicit distinct responses over a wide dynamic range of concentrations, as described for osteoblasts in the present study, may also occur in other cell types, many of which express multiple subtypes of P2 receptors. In this regard, greatly varying amounts of ATP are released into the extracellular fluid under different circumstances – smaller amounts during neurotransmission and paracrine signaling, and massive amounts in response to trauma and cell lysis (Barnes, 2007b). The presence of an ensemble of P2 receptors would allow osteoblasts and other cell types to respond appropriately in each of these different situations.

Materials and Methods

Materials and solutions
- Minimum essential medium (MEM), heat-inactivated fetal bovine serum (FBS), antibiotic solution (10,000 U/ml penicillin, 10,000 μg/ml streptomycins, and 25 μg/ml amphotericin B), trypsin solution, Dulbecco’s phosphate buffered saline (DPBS), Medium 199 buffered with HEPES (25 mM) and HCO3- (4 mM) (M199), HCO3- free MEM, Dulbecco’s modified Eagle medium (DME) (MEM) and HEPES were obtained from Gibco (Life Technologies Inc., Burlington, ON, Canada). Fluor-4 acetoxyethyl ester (fluor-4-AM), indomethacin, and Fluniconazole (F-127) were obtained from Molecular Probes (Life Technologies). FuGENE 6 and X-tremeGENE 9 were from Roche Diagnostics (Laval, QC, Canada). TRizol reagent and UltraPure distilled water (DNase/RNase-free) were obtained from Invitrogen (Life Technologies). RNeasy Mini Kit was from Qiagen (Toronto, ON, Canada). TaqMan One-Step RT-PCR Master Mix Reagents kit, COX-2 (Pigs2) primers and probe (Mm00478374_m1) and 18S rRNA primers and probe were obtained from Applied Biosystems (Life Technologies). Passive Lysis Buffer, 5× and Bright-Glo luminescence assay system were purchased from Promega (Madison, WI, USA). Biotinylated goat anti-mouse antibody, fluorescein-conjugated streptavidin (FITC) and Vectashield mounting medium with 4,6-diamidino-2-phenylindole (DAPI) were obtained from Vector Laboratories (Burlington, CA, USA). NFATc1 mouse monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Normal goat serum, collagenase type II, ethylene glycol-bis(β-aminoethylether)-N,N,N’,N’-tetraacetic acid tetrasodium salt (EGTA), adenosine 5’-triphosphate disodium salt (ATP), uridine 5’-triphosphate trisodium salt (UTP), 2′,3′-O-(4-benzoylbenzoyl)adenosine 5’-triphosphate triethylammonium salt (BzATP), 1-oleoyl-rac-glycerol-3-phosphate (LPA) and suramin sodium salt were obtained from Sigma-Aldrich (St. Louis, MO, USA). U73122 was from Enzo Life Sciences (Plymouth Meeting, PA, USA). [3H]-(5,2-Dichlorophenyl)-H-tetrazol-1-yl-methyl[phenylhydrochloride (A 438079 HC1) was from Toceis Bioscience (Ellisville, MO, USA). HEPS buffer consisted of (in mM): 135 NaCl, 5 KCl, 1 MgCl2, CaCl2, 20 HEPS and 10 glucose, adjusted to pH 7.3; 0.02, 290±5 mosmol/L CaCl2 was omitted where indicated.

Animals and cell culture
The P2X7 loss-of-function (knockout) mouse, generated as previously described (Solle et al., 2001), was obtained from Pfizer. Though P2X7 is present in this genetically modified mouse model, the protein is truncated at its C-terminus resulting in greatly diminished receptor function (Masini et al., 2012). Calvarial bone cells were isolated from 5–7-day-old mice using sequential collagenase digestion, as previously described (Panupathump et al., 2008). Freshly isolated calvarial osteoblasts were plated at a density of 1.0–1.5×106 cells/cm2 on Nun six-well plates (Thermo Fisher Scientific, Rochester, NY, USA) and maintained in α-MEM supplemented with 10% FBS and 1% antibiotic solution (culture medium) at 37°C and 5% CO2. After confluence was reached (~3–5 days), cells were trypsinized and plated for experiments. The C57BL/6 J mouse osteoblast-like cell line (subclone 4) was obtained from the American Type Culture Collection (Rockville, MD, USA). C57BL/6 J cells were cultured subtwice weekly and maintained in culture medium at 37°C and 5% CO2.

Fluorescence measurement of [Ca2+].
For confocal microscopy, M199-E1 cells or calvarial osteoblasts were plated at a density of 1.5×106 cells/cm2 on 35-mm glass-bottomed dishes (MatTek Corporation, Ashland, MA, USA) in culture medium. After 2 days, cells were plated in serum-free medium and incubated overnight. For experiments, cells were loaded with fluo-4 by incubation with fluo-4-AM (2 μg/ml) and 0.1% Pluronic F-127 for 30–45 min at 37°C and 5% CO2. Medium was then replaced with M199 supplemented with 1% antibiotic solution, and cells were observed by live-cell confocal microscopy (model LSM 510; Carl Zeiss Inc., Jena, Germany) at ~28°C using a Plan-Apochromat 40x objective (1.2 NA) with 488-nm Ar ion laser excitation. The emission wavelength was filtered at 500–550 nm and images were captured every 500 ms in time-lapse mode.

For flow cytometry, M199-E1 cells were loaded with Indo-1 as previously described (Grol et al., 2012). For measurement of [Ca2+]i, 1 ml aliquots of indo-1-loaded cell suspensions (~1×106 cells) were sedimented and resuspended in 2 ml Ca2+-containing or Ca2+-free Na+–HEPS buffer in a fluorometric cuvette at room temperature. Changes in [Ca2+]i were then monitored using a dual-wavelength spectrophotometer (Model RF-M2004; Photon Technology International, South Brunswick, NJ, USA) at 355 nm excitation and emission wavelengths of 405 and 485 nm.

Expression and localization of NFATc1–EGFP
The EGFP-tagged NFATc1 (NFATc1–EGFP) fusion protein expression vector was purchased from GE Healthcare (Amersham Place, UK). For live-cell studies, C57BL/6 J cells were plated at a density of 1.5×105 cells/cm2 on 35-mm glass-bottomed dishes in culture medium. After 1 day, cells were transfected with the NFATc1–EGFP expression vector according to manufacturer’s instructions. At 1 day post-transfection, cells were placed in serum-free medium and incubated overnight. On the day of the experiment, medium was replaced with M199 supplemented with 1% antibiotic solution, and cells were observed by confocal microscopy at ~28°C using a Zeiss Plan-Apochromat 40x objective (1.2 NA) with 488-nm Ar ion laser excitation. The emission wavelength was filtered at 500–550 nm band pass, and image stacks of 2-μm slices were captured every 5 min in time-lapse mode. Changes in subcellular localization were quantified by comparing the average fluorescence intensity in the nucleus (F505) to the average fluorescence intensity of an area of equal size in the cytosol (FCyt). Values of the ratio F505/Fcyt exceeding 1 were taken to indicate nuclear localization.

For fixed-cell studies, M199-E1 cells were plated at a density of 1.5×105 cells/cm2 on 12-mm glass coverslips in Falcon 24-well plates (BD Biosciences, Mississauga, ON, Canada) in culture medium. After 1 day, cells were transfected with the NFATc1–EGFP expression vector as described above. At 1 day
Post-transfection, cells were placed in serum-free medium and incubated overnight. On the day of the experiment, cells were incubated with test substances for the indicated times. Cells were then fixed with paraformaldehyde (4%) in sucrose solution (2%), sealed using Vectashield mounting medium with DAPI, and visualized by fluorescence microscopy. Cells were categorized as positive for nuclear localization of NFATc1–EGFP if fluorescence intensity of the nucleus exceeded that of the cytoplasm.

**Immunofluorescence localization of native NFATc1**
Calvarial osteoblasts were plated at a density of 1.5 × 10⁴ cells/cm² on Falcon 6-well plates in culture medium. After 2 days, cells were placed in serum-free medium and incubated overnight. On the day of the experiment, cells were incubated with test substances for the indicated times. Cells were then fixed with paraformaldehyde (4%) in sucrose solution (2%), permeabilized with 0.1% Triton X-100 in DPBS for 10 min, and blocked for 1 h with 1% normal goat serum in DPBS (blocking solution). To detect subcellular localization of native NFATc1, cells were incubated overnight at 4°C with a mouse monoclonal antibody (1:100 in blocking solution). The next day, cells were incubated with a biotinylated goat anti-mouse antibody (1:200 in blocking solution) for 2 h followed by 15 min incubation with fluorescein-conjugated streptavidin (1:1000 in DPBS). Stained samples were then sealed using Vectashield mounting medium with DAPI, and visualized by fluorescence microscopy. Cells were categorized as positive for nuclear localization of NFATc1 if fluorescence intensity of the nucleus exceeded that of the cytoplasm. Representative images were acquired using a Zeiss Plan-Apochromat 40× objective (1.2 NA) at a slice thickness of 2 μm with the appropriate excitation wavelengths and emission filters as described above.

**Real-time RT-PCR analyses**
MC3T3-E1 or calvarial osteoblasts were plated at a density of 1.5 × 10⁴ cells/cm² on Falcon 6-well plates in culture medium. After 2 days, cells were placed in serum-free medium and incubated overnight. On the day of the experiment, cells were incubated with test substances for the indicated times. Total RNA was isolated using TRIzol reagent and the RNeasy Mini Kit according to manufacturer’s instructions. Real-time PCR was performed using the ABI Prism 7900HT Sequence Detector (PerkinElmer) with 15 μl of passive lysis buffer, 1 μl of 1:100 dilution of the NFAT luciferase reporter vector using FuGENE 6 or X-tremeGENE 9 according to manufacturer’s instructions. Cells were subsequently plated at a density of 3.0 × 10⁴ cells/cm² on Falcon 48-well plates in culture medium. At 1 day post-transfection, cells were placed in serum-free medium and incubated overnight. On the day of the experiment, cells were treated with test substances and subsequently incubated for 24 h. Cell lysates were then prepared by incubation with 65 μl of passive lysis buffer, 1 μl per well at room temperature for a minimum of 30 min with agitation. To assess luminescence, 15 μl of lysate was combined with 15 μl of Bright-Glo Luciferase Reagent in a 96-well white plate (Greiner Bio-One, Monroe, NC, USA). Reactions for each sample were performed in triplicate. Luminescence was measured using a 2-second integration per well on a LMAX II™ microplate reader (Molecular Devices, Downingtown, PA, USA).

**Statistical analyses**
Data are shown as means±s.e.m. Differences between two groups were assessed using Student’s t tests. Differences among three or more groups were evaluated by one-way analysis of variance followed by a Tukey multiple comparisons test, or two-way analysis of variance followed by a Bonferroni multiple comparisons test. Differences were accepted as statistically significant at P<0.05.

**Acknowledgements**
We thank Dr Souzan Armstrong, Tom Chrones, Jamie Simek and Karen Nygard for assistance with confocal imaging; Andrea Lee for help with data analysis; and Kim Beauchae for constructive comments on the manuscript.


Wang, N., Oberhoffer, H., Li, Y. and Moore, S. L. (2010). The proteome of the osteoblast cell cycle: insights into the molecular events leading to bone formation. J. Proteome Res. 9, 6011-6023.
Fig. S1. Vehicle-induced elevations in $[\text{Ca}^{2+}]_i$ are suppressed by ATP. MC3T3-E1 cells were loaded with the Ca$^{2+}$-sensitive dye fluo-4 and changes in $[\text{Ca}^{2+}]_i$ were monitored by confocal microscopy. (A) and (C) Where indicated by the arrows, cells were treated with vehicle (Veh) or ATP$_{\text{low}}$ (10 μM). After ~7.5 min, the same fields of cells were treated with ATP$_{\text{low}}$ or vehicle. Pre-addition of ATP$_{\text{low}}$ suppressed Ca$^{2+}$ responses elicited by vehicle addition. Traces are representative of responses from 4 independent preparations. (B) and (D) Ca$^{2+}$ elevations were analyzed for their peak amplitude. Pre-addition of vehicle did not significantly affect subsequent responses to ATP$_{\text{low}}$ (B). In contrast, pre-addition of ATP$_{\text{low}}$ significantly suppressed subsequent responses to vehicle (D). These data suggest that small elevations in $[\text{Ca}^{2+}]_i$ induced by addition of vehicle may arise from fluid shear-induced release of ATP. α indicates significant difference between treatments ($p<0.05$). Data are means±s.e.m. ($n=8$–9 samples from 4 independent preparations).
Fig. S2. Vehicle-induced elevations in $[\text{Ca}^{2+}]_i$ are blocked by a P2 nucleotide receptor antagonist. $[\text{Ca}^{2+}]_i$ of MC3T3-E1 cells loaded with fluo-4 was monitored using confocal microscopy. (A) Cells were incubated for a minimum of 45 min in the absence (Control) or presence of the P2 receptor antagonist suramin (100 µM). Where indicated by the arrows, cells were then treated with vehicle (Veh). Suramin suppressed $\text{Ca}^{2+}$ responses elicited by vehicle addition. Traces are representative of responses from 3 independent preparations. (B) $\text{Ca}^{2+}$ elevations were analyzed for their peak amplitude. α indicates significant effect of P2 antagonist compared to control ($p<0.05$). Data are means±s.e.m. ($n=9$ samples from 3 independent preparations). Taken together with the results presented in supplemental Fig. S1, these data further support the notion that addition of vehicle creates fluid shear, leading to release of endogenous ATP and subsequent P2 receptor signaling. Consistent with this conclusion, there was virtually no $\text{Ca}^{2+}$ response to vehicle when added to cells suspended in a fluorometric cuvette with continuous stirring (Fig. 3A,D), a condition in which cells were constantly exposed to an unchanging fluid shear stimulus.
Fig. S3. The percentage of cells exhibiting P2 nucleotide receptor-induced elevations in \([\text{Ca}^{2+}]\), is dependent on ATP concentration. \([\text{Ca}^{2+}]\) of MC3T3-E1 cells loaded with fluo-4 was monitored using confocal microscopy. Concentrations of ATP indicated are final concentrations in the bath. Each concentration was tested on separate cell samples. Responses to vehicle are indicated by the letter V. (A) Data are the percentage of responding cells in a field at the indicated concentration of ATP. In this case, cells were considered to have responded when the peak amplitude (i.e. maximal rise in \([\text{Ca}^{2+}]\)) was \(\geq 100\%\) of basal levels. (B) Data are the percentage of cells in a field exhibiting a sustained \(\text{Ca}^{2+}\) response at the indicated concentration of ATP. In this case, cells were considered to have a sustained response when the amplitude at 10 min post-treatment was \(\geq 50\%\) of basal levels. Data for both (A) and (B) are means±s.e.m. \((n=8\) independent preparations). These single cell data closely resemble \(\text{Ca}^{2+}\) responses seen in whole-field analyses presented in Fig. 1C,D. Thus, the dependence of \(\text{Ca}^{2+}\) signaling on ATP concentration is due, at least in part, to changes in the proportion of responding cells.
**Fig. S4.** P2Y<sub>2</sub> and/or P2Y<sub>4</sub> mediate elevations in [Ca<sup>2+</sup>]<sub>i</sub>, elicited by ATP<sub>low</sub>, [Ca<sup>2+</sup>]<sub>i</sub>, of MC3T3-E1 cells loaded with fluo-4 was monitored using confocal microscopy. (A), (C) and (E) Where indicated by the arrows, cells were treated with ATP<sub>low</sub> (10 µM) or UTP (10 µM). After ~7.5 min, the same cells were treated again with ATP<sub>low</sub> or UTP. (B), (D) and (F) Ca<sup>2+</sup> elevations were analyzed for their peak amplitude. Pre-addition of ATP<sub>low</sub> significantly attenuated a second response to ATP<sub>low</sub> (B). Interestingly, pre-addition of UTP significantly attenuated a subsequent response to ATP<sub>low</sub> (D). Similarly, pre-addition of ATP<sub>low</sub> significantly attenuated a subsequent response to UTP (F), indicating cross-desensitization. For control experiments, pre-addition of ATP<sub>low</sub> was followed by treatment of lysophosphatidic acid (10 µM), which acts through distinct Ca<sup>2+</sup> mobilizing G protein-coupled receptors on osteoblasts (not shown). In this case, the degree of cross-desensitization was significantly less. These control data argue against the possibility that cross-desensitization of nucleotide responses is due simply to depletion of intracellular Ca<sup>2+</sup> stores. Taken together with the data presented in A-F, this suggests that ATP and UTP interact with a common receptor, likely P2Y<sub>2</sub> and/or P2Y<sub>4</sub>. Traces are representative of responses from 4 independent preparations. α indicates significant difference between treatments (p<0.05). Data are means±s.e.m. (n=8 samples from 4 independent preparations).
Fig. S5. Live-cell confocal microscopy reveals that duration of NFATc1 nuclear localization is dependent on ATP concentration. MC3T3-E1 cells were transfected with plasmids encoding EGFP-tagged NFATc1 and changes in subcellular localization of NFATc1-EGFP were monitored by confocal microscopy. (A) Representative fields of cells treated with Vehicle, ATP_low (10 μM), ATP_high (1 mM) or BzATP (300 μM). Scale bars are 20 μm. Supplemental videos 3 and 4 show responses to ATP_low and BzATP, respectively. (B) To quantify subcellular localization of NFATc1-EGFP, the average pixel intensity of the nucleus (F_N) and the average pixel intensity of an area of equal size in the cytosol (F_C) were determined. Values of the ratio F_N/F_C were plotted as a function of time, and values for F_N/F_C exceeding 1 were taken to indicate nuclear localization. Plots are representative time courses of nuclear localization for the cells marked with a white asterisk in (A). The time of addition of test substance is indicated by the vertical broken line. Data are representative responses of cells from a minimum of 4 independent transfections.
Fig. S6. P2 nucleotide receptor-induced changes in NFAT transcriptional activity are mediated by Ca\(^{2+}\)-calcineurin signaling. (A) MC3T3-E1 cells were incubated for 30 min in the absence or presence of calcineurin inhibitors cyclosporin A (1 μM) or FK506 (1 μM). Next, cells were treated for 3 h with vehicle, ATP\(_{\text{low}}\), ATP\(_{\text{high}}\) or BzATP in the continued absence (Control) or presence of inhibitor. Total RNA was then isolated and real-time RT-PCR was performed to assess expression levels of *Ptgs2*. Data are normalized to 18S and expressed relative to values for control cultures treated with vehicle. α indicates a significant difference from vehicle; β indicates significant difference between ATP\(_{\text{high}}\) or BzATP and ATP\(_{\text{low}}\); δ indicates significant effect of inhibitor (p<0.05). Data are means±s.e.m. (n=6 samples from 3 independent preparations). (B) MC3T3-E1 cells transfected with the NFAT luciferase reporter plasmid were incubated for 30 min in the absence (Control) or presence of cyclosporin A (1 μM) or FK506 (1 μM). Next, cells were treated with vehicle, ATP\(_{\text{low}}\), ATP\(_{\text{high}}\) or BzATP in the continued absence or presence of inhibitor. After 24 h, cell lysates were collected and luminescence was assessed. Luminescence was expressed relative to values for control cultures treated with vehicle. α indicates significant difference from vehicle; β indicates significant difference between ATP\(_{\text{high}}\) or BzATP and ATP\(_{\text{low}}\) (p<0.05). Data are means±s.e.m. (n=9 samples from 3 independent preparations).
Movie 1. A low concentration of ATP (10 μM) elicits a transient increase in cytosolic free Ca$^{2+}$. MC3T3-E1 cells were loaded with the Ca$^{2+}$-sensitive dye fluo-4 and changes in [Ca$^{2+}$] were monitored by live-cell confocal microscopy under serum-free conditions. Cultures were bathed in M199 supplemented with 1% antibiotic solution at ~25 °C and ATP (10 μM; ATP$_{low}$) was added at 10 min. Movie begins at 0 min and ends at 30 min real-time. Image intervals are 1.5 s and frames are shown at 100 frames/s. Width of the field is 230 μm.

Movie 2. A high concentration of ATP (1 mM) elicits a sustained increase in cytosolic free Ca$^{2+}$. MC3T3-E1 cells were loaded with the Ca$^{2+}$-sensitive dye fluo-4 and changes in [Ca$^{2+}$] were monitored by live-cell confocal microscopy under serum-free conditions. Cultures were bathed in M199 supplemented with 1% antibiotic solution at ~25 °C and ATP (1 mM; ATP$_{high}$) was added at 10 min. Movie begins at 0 min and ends at 30 min real-time. Image intervals are 1.5 s and frames are shown at 100 frames/s. Width of the field is 230 μm.
Movie 3. A low concentration of ATP (10 μM) elicits NFATc1-EGFP nuclear translocation of transient duration. MC3T3-E1 cells were transfected with plasmids encoding EGFP-tagged NFATc1 and changes in subcellular localization of NFATc1-EGFP were monitored by live-cell confocal microscopy under serum-free conditions. Cultures were bathed in M199 supplemented with 1% antibiotic solution at ~25 °C and ATP (10 μM; ATP
low) was added at 12.5 min. Movie begins at 0 min and ends at 197 min real-time. Image intervals are 2.5 min and frames are shown at 10 frames/s. Width of the field is 230 μm.

Movie 4. The P2X7 agonist BzATP elicits NFATc1-EGFP nuclear translocation of sustained duration. MC3T3-E1 cells were transfected with plasmids encoding EGFP-tagged NFATc1 and changes in subcellular localization of NFATc1-EGFP were monitored by live-cell confocal microscopy under serum-free conditions. Cultures were bathed in M199 supplemented with 1% antibiotic solution at ~25 °C and BzATP (300 μM) was added at 12.5 min. Movie begins at 0 min and ends at 194 min real-time. Image intervals are 2.5 min and frames are shown at 10 frames/s. Width of the field is 154 μm.