High-affinity P2Y<sub>2</sub> and low-affinity P2X<sub>7</sub> receptor interaction modulates ATP-mediated calcium signaling in murine osteoblasts

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Abstract

The P2 purinergic receptor family implicated in many physiological processes, including neurotransmission, mechanical adaptation and inflammation, consists of ATP-gated non-specific cation channels P2XRs and G-protein coupled receptors P2YRs. Different cells, including bone forming osteoblasts, express multiple P2 receptors; however, how P2X and P2Y receptors interact in generating cellular responses to various doses of [ATP] remains poorly understood. Using primary bone marrow and compact bone derived osteoblasts and BMP2-expressing C2C12 osteoblastic cells, we demonstrated conserved features in the P2-mediated Ca<sup>2+</sup> responses to ATP, including a transition of Ca<sup>2+</sup> response signatures from transient at low [ATP] to oscillatory at moderate [ATP], and back to transient at high [ATP], and a non-monotonic changes in the response magnitudes which exhibited two troughs at 10<sup>−4</sup> and 10<sup>−2</sup> M [ATP]. We identified P2Y<sub>2</sub> and P2X<sub>7</sub> receptors as predominantly contributing to these responses and constructed a mathematical model of P2Y<sub>2</sub>R-induced inositol trisphosphate (IP<sub>3</sub>) mediated Ca<sup>2+</sup> release coupled to a Markov model of P2X<sub>7</sub>R dynamics to study this system. Model predictions were validated using parental and CRISPR/Cas9-generated P2Y<sub>2</sub> and P2Y<sub>7</sub> knockouts in osteoblastic C2C12-BMP cells. Activation of P2Y<sub>2</sub> by progressively increasing [ATP] induced a transition from transient to oscillatory to transient Ca<sup>2+</sup> responses due to the biphasic nature of IP<sub>3</sub>Rs and the interaction of SERCA pumps with IP<sub>3</sub>Rs. At high [ATP], activation of P2X7R modulated the response magnitudes through an interplay between the biphasic nature of IP<sub>3</sub>Rs and the desensitization kinetics of P2X7Rs. Moreover, we found that P2Y2 activity may alter the kinetics of P2X7 towards favouring naïve state activation. Finally, we demonstrated the functional consequences of lacking P2Y2 or P2X7 in osteoblast mechanotransduction. This study thus provides important insights into the biophysical mechanisms underlying ATP-dependent Ca<sup>2+</sup> response signatures, which are important in mediating bone mechanoadaptation.
Author summary

ATP-sensitive purinergic receptors comprise a network of cell-surface receptors that activate upon ATP binding, allowing them to transmit information in a tissue- and context-dependent manner. In bone, mechanically stimulated osteoblasts release ATP that stimulates low- and high-affinity P2 receptors in neighboring cellular populations, inducing appropriate physiological responses. P2 receptor signaling is characterized by elevations in intracellular calcium levels. When simultaneously stimulated by their common ligand, ATP, the contribution of each P2 receptor subtype gives rise to a complex calcium response, exhibiting oscillatory characteristics and biphasic dose-dependent behaviours. Here we used experimental and computational modeling approaches to determine the underlying dynamics of ATP-mediated calcium signaling in osteoblasts. The latter was done by developing a mathematical model that was comprised of a subset of low- (P2X7) and high- (P2Y2) affinity P2 receptors, reflecting the conserved P2 expression observed across different osteoblast models. We demonstrated that this model recapitulates experimental recordings of ATP-induced calcium signaling in osteoblasts and describes the dynamic interplay between P2Y2 and P2X7 receptors in the P2 receptor network.

Introduction

Extracellular ATP has long been implicated in diverse physiological functions [1], including neurotransmission [2], mechanical adaptation [3] and the regulation of inflammation [4]. Extracellular purines signal through 7 ionotropic receptors, i.e., the P2X ligand-gated nonspecific cation channels, and 8 metabotropic receptors, i.e., the P2Y G-protein coupled receptors [5].

ATP is the physiological agonist for all P2X receptors (P2XRs) as well as the P2Y2 and P2Y11 receptors (P2Y2R and P2Y11R, respectively) [6]. Together they cover a range of extracellular ATP concentration ([ATP]) spanning six orders of magnitude (10^{-8} M to 10^{-2} M) [7]. P2XRs are fast acting (~10 ms activation), allowing the permeation of Na\(^+\), K\(^+\) and Ca\(^{2+}\) through the channel [8] whereas P2YRs activate various types of secondary messengers, and thus act on a slower timescale than P2XRs [9]. Elevations in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) is one of the hallmarks of ATP-induced signaling in many cell types, including bone-forming osteoblasts [7,10–13]. The mechanism by which P2XRs and P2YRs alter [Ca\(^{2+}\)]\(_i\) differs: P2XR activation increases Ca\(^{2+}\) influx across the plasma membrane [14] while P2YR activation enhances Ca\(^{2+}\) release from the endoplasmic reticulum (ER) by stimulating the G\(_q\) protein signaling pathway, ultimately leading to the production of inositol triphosphate (IP\(_3\)) and the activation of IP\(_3\) receptors (IP\(_3\)Rs) [15]. The ATP dose dependence of osteoblast responses to [ATP] was shown to be complex and does not have a clear plateau component, an outcome not explainable by the addition of individual receptor responses [7]. While it was proposed that specific interactions between the high-affinity, mid-range and low-affinity P2Rs may explain the [ATP]-dependence, no mechanistic studies at the level of cellular signaling has yet been performed.

Markov models of P2X2/4/7R were previously developed to decipher the kinetics of ATP binding to these receptors and illustrate the interplay between receptor activation, priming, desensitization, internalization and deactivation [16–21]. Mathematical modeling has similarly been used to provide insights into the P2Y receptor signaling, particularly in the regulation of IP\(_3\)R-mediated Ca\(^{2+}\) release [22,23]. However, how P2X and P2Y receptors interact and what are their respective roles in generating cellular responses to various doses of [ATP] remains poorly understood.
In this study, we combined detailed experimental and computational studies of ATP-induced Ca\(^{2+}\) signals in primary mouse osteoblasts and BMP2-transfected C2C12 osteoblastic cells. We demonstrated the specific contributions of P2Y2 and P2X7 receptors to global Ca\(^{2+}\) responses using CRISPR/Cas9 -generated P2Y2 and P2Y7 knockouts in osteoblastic C2C12-BMP cell lines, and dissected the mechanisms of P2Y2 and P2X7 contributions to generating different patterns of oscillatory and sustained Ca\(^{2+}\) signals using mathematical modeling.

**Results**

**ATP-mediated P2R Ca\(^{2+}\) responses in murine osteoblasts**

ATP-stimulated P2R Ca\(^{2+}\) responses were assessed in three independent murine osteoblasts models: BMP2-transfected C2C12 osteoblastic cells (C2-OB), bone-marrow-derived osteoblasts (BM-OB), and compact-bone-derived osteoblasts (CB-OB). Osteoblasts were loaded with Ca\(^{2+}\)-indicator dye Fura2, stimulated with varying doses of ATP, and changes in [Ca\(^{2+}\)]\(_i\) were recorded using live cell fluorescent microscopy (Fig 1). Qualitatively, the recorded Ca\(^{2+}\) response time-series signatures demonstrated a general trend of exhibiting transient single-peaked responses at low [ATP], multi-peaked oscillatory responses at mid-range [ATP], and relatively sustained single/multi-peaked response at high [ATP] (Fig 1A). The Ca\(^{2+}\) responses at each [ATP] were analysed by quantifying several parameters, including overall response magnitudes and activation times, as well as oscillatory fractions, magnitudes, periods and peaks (see S1 Table for definitions) [13]. Similar to previous study [7], we harmonized dose-response profiles across osteoblast models by first aligning the responses along the dose-axis to match troughs/peaks, followed by rescaling the responses to a [0,1] interval (S1 Fig). Such alignment allowed us to account for (i) inconsistencies in ATP solution preparations between experiments and (ii) varied dose-sensitivities across cell lines. Calcium responses induced by low [ATP] (<10\(^{-7}\) M) were consistently associated with low response magnitudes and slow activation kinetics (Fig 1A, left two columns), with little to no oscillatory component (Fig 1B). Increasing [ATP] further induced responses with faster activation kinetics and higher magnitudes (Fig 1A, middle two columns). This also coincided with the emergence of high frequency oscillations (~10–20 s periods; Figs 1B and S1B and S1C) which peaked at ~10\(^{-5}\) M ATP stimulation. Notably, the oscillatory peak did not coincide with the peak magnitude. Instead, as cells were stimulated with higher [ATP], the oscillatory component began to diminish, exhibiting lower frequency oscillations and fewer oscillatory peaks, while response magnitude continued to increase, peaking at ~2\times10\(^{-4}\) M [ATP] (Fig 1A, right two columns, and 1B). For [ATP] >2\times10\(^{-4}\) M, the response magnitude decreased with increasing [ATP] in all osteoblast lines. Thus, in all osteoblast models, the intracellular Ca\(^{2+}\) response to ATP shifts with increase in [ATP] from a transient with a single narrow peak, to oscillatory and back to transient with a pronounced wide peak.

**P2Y\(_2\) and P2X\(_7\) receptors orchestrate the ATP-mediated Ca\(^{2+}\) responses**

To examine which P2 receptors contribute to the ATP-induced Ca\(^{2+}\) responses, we first examined their expression in osteoblastic cells of different origin. Among the P2Y family, P2ry2, P2ry4, P2ry12, and P2ry14 transcripts were detected in all osteoblastic cells by RT-qPCR (Fig 2A top). Among the P2X family, P2rx4 and P2rx7 transcripts were the most abundantly expressed in all osteoblastic cells (Fig 2A bottom). These expression profiles suggest that P2Y2, 4, 12, 14 and P2X4, 7 are the predominant P2 receptor subtypes expressed in osteoblastic cells, among which P2Y2 and P2X7 were the most abundant transcripts. To confirm that P2Y2 and P2X7 receptors are functional, we stimulated Fura2-loaded C2-OB cells with ATP and receptor-specific agonists: the P2Y2-agonist UTP and P2X7-agonist bzATP (Fig 2B). Consistent with previously characterized P2 receptor sensitivities [7], we found that the estimated EC50s...
were 1.0 μM for ATP, 2.8 μM for UTP and 26.4 μM for bzATP in C2-OB cells. Importantly, the oscillatory Ca$^{2+}$ responses evoked by 10^{-6} M ATP were recapitulated following stimulation with 10^{-6} M UTP, and similarly, the sustained responses evoked by 1 mM ATP were observed following 1 mM bzATP stimulation, suggesting that P2Y2 and P2X7 receptors dominate the responses to lower and higher [ATP], respectively (Fig 2C). Using CRISPR-Cas9 double-nickase constructs, we generated clonal C2-OB cells harboring mutations in $P2ry2$ ($P2ry2$Δ) or $P2rx7$ ($P2rx7$Δ) (Fig 2D) to further investigate the independent contribution of P2Y2 and P2X7 to the P2-mediated Ca$^{2+}$ responses (presented in subsequent sections).

A flux-balance-based model of P2Y2R and P2X7R driven Ca$^{2+}$ responses

To decipher the underlying biophysical mechanisms governing the ATP-stimulated Ca$^{2+}$ responses in osteoblasts, we developed a mathematical model that integrates P2Y2R-mediated
Ca\textsuperscript{2+} release, as described by the Li-Rinzel model of the IP\textsubscript{3}R [24] with a Markov model of P2X7R kinetics adapted from [18] (Fig 3). The cell was divided into two compartments (Fig 3A), the endoplasmic reticulum (ER) and the cytosol, with Ca\textsuperscript{2+} concentrations in each compartment denoted by [Ca\textsuperscript{2+}]\textsubscript{ER} and [Ca\textsuperscript{2+}]\textsubscript{i}, respectively. The detailed description of the model is given in Methods. Briefly, the model describes (i) Ca\textsuperscript{2+} mobilization across cell membrane, including Ca\textsuperscript{2+} influx through P2X7R receptor channels (\(J_{P2X7}\)) and the constant inward leak (\(J_{INleak}\)), as well as Ca\textsuperscript{2+} efflux through the plasma-membrane-Ca\textsuperscript{2+}-ATPase (PMCA) pumps (\(J_{PMCA}\)); and (ii) Ca\textsuperscript{2+} mobilization across ER membrane, including P2Y2R-mediated Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}-release (CICR) through IP\textsubscript{3}R (\(J_{IP3R}\)), Ca\textsuperscript{2+} leak across the ER membrane (\(J_{ER-leak}\)), and Ca\textsuperscript{2+} uptake through the sarco/endoplasmic Ca\textsuperscript{2+} ATPase (SERCA) pumps (\(J_{SERCA}\)). The reduced two-dimensional Li-Rinzel model for IP\textsubscript{3}R-mediated CICR was chosen for its simplicity and ability to produce transitions between the desired modes of activity; it follows the Hodgkin-Huxley gating formalism (see Methods), with two fast activation variables and one slow inactivation variable that depend on [IP\textsubscript{3}] and [Ca\textsuperscript{2+}]\textsubscript{i}, producing an open probability profile for CICR that is biphasic with respect to [Ca\textsuperscript{2+}]\textsubscript{i}. Given that P2Ys modulate intracellular Ca\textsuperscript{2+} responses indirectly by stimulating IP\textsubscript{3} production, an equation describing [ATP]-dependent IP\textsubscript{3} production was added to the P2Y2R submodel.

Ca\textsuperscript{2+} flux through P2X7R, \(J_{P2X7}\), on the other hand, was determined by the Ca\textsuperscript{2+} current (\(I_{P2X7}\)) through the receptor channels generated by a 12-state Markov P2X7R sub-model.

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Fig 2. Functional P2Y\textsubscript{2} and P2X\textsubscript{7} are expressed in osteoblastic cells. (A) P2 expression determined by RT-qPCR in C2-OB, BM-OB, and CB-OB. Relative transcript expression was calculated by ΔΔCT method, and P2ry2 and P2rx4 were used as calibrators for P2Y and P2X receptors, respectively. Data are means ± SEM, n = 3 independent cultures per cell line. (B) Fura2-loaded C2-OB cells were stimulated by ATP, UTP, or BzATP and [Ca\textsuperscript{2+}] responses magnitudes were measured. Data are normalized means ± SEM (markers) fitted with hill functions (curves) for their dose-response curves. (C) Representative Ca\textsuperscript{2+} responses observed in C2-OB stimulated by 10\textsuperscript{-8} M ATP or UTP, and 10\textsuperscript{-3} M ATP or BzATP. (D) P2Y\textsubscript{2} and P2X\textsubscript{7} protein expression assessed by immunoblot in WT, P2ry2Δ and P2rx7Δ C2-OB whole cell lysates. Histone H3 was used as a loading control.

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The P2X7R submodel assumes that each receptor has three ATP binding sites, two of which must be occupied for the receptor to be open, and that each state represents the fraction of receptors with a given number of occupied ATP-binding sites (Fig 3B, solid circles). The closed, $C_i$, and desensitized, $D_i$, states are non-conducting, whereas the open states $Q_i$ ($i = 1−4$) possess the same conductance $g_{X7}$. The states were divided into three rows corresponding to desensitized (Fig 3B, top row), naïve (Fig 3B, middle row) and sensitized or primed (Fig 3B, bottom row) states, respectively. The naïve row is comprised of the states $C_1$, $C_2$, $Q_1$, $Q_2$ that have not been exposed to ATP for a prolonged period of time, whereas the sensitized and desensitized rows are comprised of the states $C_3$, $C_4$, $Q_3$, $Q_4$ ($D_1$, $D_2$, $D_3$, $D_4$) that...
have been previously exposed to ATP. The forward and backward transitions along each row describes ATP binding and unbinding, respectively, whereas downward and upward transitions between the rows represent receptor sensitization (middle to bottom row), desensitization (middle to top row) or recovery (bottom/top to middle row). The rate of desensitization increases as more ATP molecules bind to P2X7R and the open probability along the sensitized row is larger than that for the naïve row.

Combining the two submodels together produced the following model:

\[
\frac{d[Ca^{2+]}}{dt} = f_c(J_{P2X7} + J_{P2Y2} - J_{PMCA} + J_{IP3R} + J_{ERCa})
\]

\[
\frac{d[Ca^{2+}]_{ER}}{dt} = f_{ER}J_{SERCA} - J_{IP3R} + J_{ERCa}
\]

\[
\frac{d[IP_3]}{dt} = \alpha_{ATP} \frac{[ATP]}{[ATP] + k_{ATP}} - \delta_{ATP}[IP_3]
\]

where \( f_c \) and \( f_{ER} \) represent the fraction of free Ca\(^{2+}\) in the cytosol and ER, respectively, as a result of buffering with \( 0 < f_c, f_{ER} < 1 \), \( \gamma \) is the ratio between cytosolic and ER volume, \( \alpha_{ATP} \) is the maximum rate of IP\(_3\) production by P2Y2 in response to ATP, \( k_{ATP} \) is the half-maximum production of IP\(_3\) through P2Y2R and \( \delta_{ATP} \) is the rate of IP\(_3\) degradation.

Using the model parameters provided in Table 1, we simulated Ca\(^{2+}\) responses to different [ATP] in three specific cases: (i) in naïve cells expressing both P2Y2R and P2X7R using the full model (Fig 4A, blue curves), (ii) in cells that do not express P2Y2R, using the submodel for P2X7 component only (Fig 4A, grey curves), and (iii) in cells that do not express P2X7R using the submodel for P2Y2 component only (Fig 4A, yellow curves). The simulated Ca\(^{2+}\) responses were compared with those obtained experimentally in WT (Fig 4B, blue curves), P2ry2Δ (Fig 4B, grey curves) and P2rx7Δ (Fig 4B, yellow curves) C2-OB cells. As shown, the responses to low [ATP] were predominantly P2Y2-mediated, while the response to high [ATP] were jointly mediated by P2Y2 and P2X7. Notably, the characteristic two-peaked response to 10^{-3} M observed in experimental recordings (Figs 1A and 4B) was predicted by the full model (Fig 4A) of WT cells, but was abolished in P2ry2Δ and P2rx7Δ recordings and in simulations of P2X7 and P2Y2 submodels. These data strongly support the interaction between P2Y2 and P2X7 receptors in generating this unique signaling feature.

One interesting aspect of the recordings and simulations displayed in Fig 4 was the observation of oscillatory Ca\(^{2+}\) responses at intermediate [ATP], with transient responses at low and high [ATP], indicating that the mathematical model developed in this study recapitulates the characteristic Ca\(^{2+}\) signatures observed in C2-OB cells over a physiological range of [ATP].

**P2Y2 drives the transition from transient to oscillatory Ca\(^{2+}\) responses**

Since the oscillatory component required P2Y2 activity in both experimental and simulated responses to ATP, we next investigated how the transition between transient and oscillatory responses is achieved by the P2Y2 receptor. Given that [IP\(_3\)] and [Ca\(^{2+}\)]\(_{ER}\) are slow variables in the model defined by Eqs (1)–(3), we set \( J_{P2X7R} = 0 \) and applied slow-fast analysis on the resulting P2Y2 receptor model by assuming that these two variables change slowly relative to other “fast” variables in the model. We set the two variables ([IP\(_3\)] and [Ca\(^{2+}\)]\(_{ER}\)) as independent adjustable parameters in the P2Y2 model and investigated how the steady state dynamics of fast variables change when [IP\(_3\)] and [Ca\(^{2+}\)]\(_{ER}\) are altered. The two-parameter bifurcation diagram (Fig 5) exhibited an oscillatory region in the “parameter” space formed by [IP\(_3\)] and [Ca\(^{2+}\)]\(_{ER}\).
### Table 1. Mathematical Model Parameters.

#### Buffering & Scaling

| Symbol | Value | Description | Reference |
|--------|-------|-------------|-----------|
| \( f_i \) | 0.01 | Cytosolic \( \text{Ca}^{2+} \) buffering | [38] |
| \( F_{ER} \) | 0.025 | ER \( \text{Ca}^{2+} \) buffering | [38] |
| \( \gamma \) | 9 | cytosol/ER volume ratio | [39] |

#### Plasma Membrane Fluxes

| Symbol | Value | Description | Reference |
|--------|-------|-------------|-----------|
| \( J_{INleak} \) | 0.15 \( \mu \text{Ms}^{-1} \) | Inward leak across plasma membrane | Fitted† |
| \( v_{PMCA} \) | 30 \( \mu \text{Ms}^{-1} \) | Maximum PMCA activation | Fitted† |
| \( k_{PMCA} \) | 0.45 \( \mu \text{M} \) | PMCA \( \text{Ca}^{2+} \) affinity | [32] |

#### ER Fluxes

| Symbol | Value | Description | Reference |
|--------|-------|-------------|-----------|
| \( v_{SERCA} \) | 22.5 \( \mu \text{Ms}^{-1} \) | Maximum SERCA activation | Fitted† |
| \( k_{SERCA} \) | 0.105 \( \mu \text{M} \) | SERCA \( \text{Ca}^{2+} \) affinity | [32] |
| \( v_{ERleak} \) | 0.03 \( \text{s}^{-1} \) | Rate of \( \text{Ca}^{2+} \) leak across ER membrane | Fitted† |

#### Li Rinzell IP\( _3 \)R Models

| Symbol | Value | Description | Reference |
|--------|-------|-------------|-----------|
| \( d_1 \) | 0.13 \( \mu \text{M} \) | IP\( _3 \) dissociation constant (\( \text{Ca}^{2+} \) unbound from inactivation site) | [24] |
| \( d_2 \) | 1.049 \( \mu \text{M} \) | \( \text{Ca}^{2+} \) dissociation constant from the inactivation site (IP\( _3 \) bound) | [24] |
| \( d_3 \) | 0.9434 \( \mu \text{M} \) | IP\( _3 \) dissociation constant (\( \text{Ca}^{2+} \) bound to inactivation site) | [24] |
| \( d_4 \) | 0.08234 \( \mu \text{M} \) | \( \text{Ca}^{2+} \) dissociation constant from activation site | [24] |
| \( a_2 \) | 0.2 \( \mu \text{Ms}^{-1} \) | \( \text{Ca}^{2+} \) binding rate to the inactivation site | [24] |
| \( v_{IPR} \) | 15 \( \mu \text{Ms}^{-1} \) | Maximum flux through IP\( _3 \)R | Fitted† |

#### P2X7R Model

| Symbol | Value | Description | Reference |
|--------|-------|-------------|-----------|
| \( g_X \) | 2.5x10\( ^8 \) \( \text{Ms}^{-1} \) | P2X7R conductance of both naive and sensitized open states | Adjusted*** |
| \( E \) | 0 mV | Reversal potential | [18] |
| \( k_1 \) | 0.3 \( \text{s}^{-1} \) | Transition rates between states (Fig 3B, along the same row) | [18]* |
| \( k_2 \) | 1265 M\( ^{-1} \text{s}^{-1} \) | | [18]* |
| \( k_3 \) | 2.4 \( \text{s}^{-1} \) | | [18]* |
| \( k_4 \) | 1581 M\( ^{-1} \text{s}^{-1} \) | | [18]* |
| \( k_5 \) | 1.58 \( \text{s}^{-1} \) | | [18]* |
| \( k_6 \) | 221 M\( ^{-1} \text{s}^{-1} \) | | [18]* |
| \( k_7 \) | 316 M\( ^{-1} \text{s}^{-1} \) | | [18]* |
| \( L_1 \) | 0.0001 | Transition rates between naïve (Fig 3B, middle row) and sensitized (Fig 3B, bottom row) | [18] |
| \( L_2 \) | 0.004 | | [18] |
| \( L_3 \) | 0.3 | | [18] |
| \( H_1 \) | 0.001 | Transition rates between naïve (Fig 3B, middle row) and desensitized (Fig 3B, upper row) | [18] |
| \( H_{2(C2)} \) | 0.01 | | [18] |
| \( H_{2(Q1)} \) | 0.05 | | Adjusted*** |
| \( H_{2(Q2)} \) | 0.8 | | Adjusted*** |
| \( V \) | -0.06 V | Membrane Potential | [18] |
| \( F_{Ca} \) | 0.046 | Fraction of P2X7R flux that is \( \text{Ca}^{2+} \) | [36] |
| \( V_{oste} \) | 6.5 \( \text{pL} \) | Osteoblast volume | [35,40] |

#### IP\( _3 \) Dynamics

| Symbol | Value | Description | Reference |
|--------|-------|-------------|-----------|
| \( \alpha_{ATP} \) | 0.03 \( \mu \text{Ms}^{-1} \) | Maximum rate of IP\( _3 \) production driven by ATP | [30,41]** |
| \( k_{ATP} \) | 1 \( \mu \text{M} \) | Sensitivity of IP\( _3 \) production to [ATP] | Fitted† |

(Continued)
Table 1. (Continued)

| δ    | 0.01 s⁻¹ | Degradation rate of IP₃ | [30,41]²³

* Parameter values in [18] were fitted to BzATP. To capture the lower binding affinity of ATP to P2X7R, these parameter values were rescaled here (by dividing them by the factor 31.625).

** Parameter values from [30] were used as an upper bound for the parameters in this work, based on the significantly slower IP₃ dynamics reported in living cells in [41].

*** As stated in the text, these values were adjusted due to the evidence that P2X7R do not dilate and that P2X7R desensitization increases with more ATP binding.

† These parameter values were determined by fitting the model to data of dose-responses of ATP-induced [Ca²⁺]ᵢ elevations shown in Fig 7.

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Fig 4. Comparison of simulated and experimental dynamics of ATP-induced [Ca²⁺]ᵢ responses. (A) Time series simulations of [Ca²⁺]ᵢ responses generated by the complete model of P2Y₂ and P2X₇ (full model, blue), P2X₇ submodel (grey) and P2Y₂ submodel (yellow). Parameter values are provided in Table 1. (B) Experimental recordings of [Ca²⁺]ᵢ responses in WT (blue), P2y2Δ (grey) and P2x7Δ (yellow) C2-OB cells in response to varying [ATP].

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transition between these two main patterns of activity: quiescence and oscillatory. Thus the application of increasing [ATP] in this model would induce an increase in IP$_3$ (since [IP$_3$/[ATP]]) and a decrease in [Ca$^{2+}$]$_{ER}$, generating three possible scenarios for the time courses of [Ca$^{2+}$]$_i$.

When low [ATP] is applied the trajectory stays to the left of the oscillatory region (Fig 5A, red arrow) because the ATP-induced IP$_3$ increase is low (Fig 5B, red curve) and [Ca$^{2+}$]$_{ER}$ remains high (Fig 5C, red curve), resulting in a low magnitude persistent transient response (Fig 5D, red curve). Intermediate [ATP] results in a hybrid response that becomes periodic when the trajectory crosses the left boundary of the oscillatory regime (Fig 5A, green curve), due to a higher increase in IP$_3$ and a faster decrease in [Ca$^{2+}$]$_{ER}$ (Fig 5B and 5C, green curves), leading to a response characterized by damped oscillations (Fig 5D, green curve). When high [ATP] is applied, the trajectory crosses the oscillatory region very briefly (Fig 5A, blue curve) because even though the ATP-induced IP$_3$ increase is higher (Fig 5B, blue curve), the [Ca$^{2+}$]$_{ER}$ does not decrease as fast (Fig 5C, blue curve) due to the biphasic nature of IP$_3$Rs incorporated in the P2Y2 sub-model and the interaction of SERCA pumps with IP$_3$Rs [24]; this results in a high magnitude semi-persistent response (Fig 5D, blue curve). The aforementioned mechanism suggests that the heterogeneity in Ca$^{2+}$ response profiles at a given [ATP] observed experimentally (Fig 1A) may be due to variations in the initial conditions, such as the expression levels of PMCA/SERCA pumps and IP$_3$Rs, potentially leading to differences in the initial [IP$_3$] and [Ca$^{2+}$]$_{ER}$. These simulations demonstrate that the oscillatory response strongly depends on the initial [IP$_3$] and [Ca$^{2+}$]$_{ER}$, and that when increasing doses of [ATP] are applied, P2Y2-mediated changes in [IP$_3$] and [Ca$^{2+}$]$_{ER}$ result in different patterns of Ca$^{2+}$ responses.

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P2X7 modulates the magnitude of Ca$^{2+}$ response to ATP

We next investigated the non-monotonic $[\text{ATP}]$-dependent dose-response profile for the magnitude of Ca$^{2+}$ response observed across all osteoblastic lines (Fig 1B). Using the model defined by Eqs (1)–(3), we generated Ca$^{2+}$ responses to $[\text{ATP}]$ ranging between $10^{-8}$ to $10^{-2}$ M ATP (with $10^{-8}$ M $[\text{ATP}]$ increments), and computed the maximum $[\text{Ca}^{2+}]_i$ reached within 120 s (Fig 6A). The full model (Fig 6A, blue curve) recapitulated the experimental magnitude dose-response profile (Fig 6A blue triangles), including the troughs at moderate (Fig 6A, light grey region) and elevated (Fig 6A, dark grey region) $[\text{ATP}]$ within the plateau component of the response. This dose-dependency became monotonic in experimental recordings of Fura2-loaded P2rx7Δ C2-OB cells stimulated with ATP (Fig 6A, red circles) and in the model lacking P2X7Rs (Fig 6A, red curve), strongly implicating P2X7 in this phenomenon. Therefore, we next plotted the total Ca$^{2+}$ flux through P2X7Rs, estimated as the area under the P2X7R flux curve, as well as the maximal Ca$^{2+}$ fluxes through P2X7Rs and IP$_3$Rs predicted by the model (Fig 6B–6E). When P2X7R-mediated Ca$^{2+}$ entry became evident at $10^{-5}$ M ATP (Fig 6B, light grey region), the maximum flux through IP$_3$Rs in the full model (Fig 6C, black curve) dropped below that of the P2X7R-lacking submodel (Fig 6C, red curve) due to the biphasic dependence of IP$_3$Rs on $[\text{Ca}^{2+}]_i$, resulting in the first trough in the dose-response (Fig 6A, light grey region). At high $10^{-2}$ M ATP, on the other hand, the time required for the P2X7R flux to decay to half of its maximum ($t_{1/2}$) decreased (Fig 6D). As a result, despite the maximum P2X7R flux continually increasing (Fig 6E), the Ca$^{2+}$ entering through P2X7R began to decrease at elevated $[\text{ATP}]$ (Fig 6B, dark grey), resulting in the second trough in the dose-response (Fig 6A, dark grey region).
Taken together, these simulations indicate that the non-monotonic $Ca^{2+}$ dose-response to ATP is driven by an interplay between the biphasic nature of $IP_3$Rs and the desensitization kinetics of $P2X7$Rs.

**Contribution of $P2Y2$ to $Ca^{2+}$ responses at high [ATP]**

Next, we examined why the $Ca^{2+}$ response to high [ATP] is dramatically affected in $P2ry2\Delta$ cells (Figs 4B and 7A). We examined the magnitudes of $Ca^{2+}$ responses to $10^{-2}$ M [ATP] in WT $P2ry2\Delta$ cells, and found that in the absence of $P2Y2R$, response magnitudes exhibited a distinct bimodal distribution with one cluster of responses similar to those in WT, and another one with much higher response magnitudes (Fig 7B). We hypothesized that the bimodality in the $P2X7R$-mediated responses is due to $P2X7R$s being in the naïve or sensitized initial state [20]. To verify this, we used the $P2X7$ submodel to simulate the $Ca^{2+}$ response in two different scenarios: initiating the $P2X7$-simulations from the naïve closed state $C_1$ (i.e., $C_i(0) = 1$, $C_i(0) = 0$, for $i = 2–4$, and $Q_i(0) = D_i(0) = 0$, for $i = 1–4$), or from the sensitized closed state $C_4$ (i.e., $C_4(0) = 1$, $C_i(0) = 0$, for $i = 1–3$, and $Q_i(0) = D_i(0) = 0$, for $i = 1–4$). These two scenarios reflect the heterogeneity in the distribution of unstimulated $P2X7R$ as being predominantly in the naïve or sensitized states. Plotting the [ATP]-dependent dose-response curve for $[Ca^{2+}]_i$ generated from these time series simulations revealed that $P2X7R$ responses initiated from $C_1$ produced a dose-response curve that plateaued at a depressed level of $[Ca^{2+}]_i$ (≈ 40% of max WT response; Fig 7A, solid red curve), close to the mean of the left mode of the distribution (Fig 7B). In contrast, $P2X7R$ responses initiated from $C_4$ state produced a dose-response curve that plateaued at an elevated $[Ca^{2+}]_i$ (≈ 200% of max WT response; Fig 7A, dashed red curve), closely matching the mean of the right mode of the distribution (Fig 7B). These data suggest that $P2Y2$ activation may alter the kinetics of $P2X7$ towards favouring naïve state activation.

**Fig 7. $P2Y2$ alters $P2X7$-mediated $[Ca^{2+}]_i$ response to high [ATP].** (A) The magnitude dose-responses of ATP-induced $[Ca^{2+}]_i$ elevations. Markers indicate experimental means ± SEM in wild-type (WT; blue) and $P2ry2\Delta$ (red) C2-OB cells. Curves indicate simulated data generated by the full model (blue), a $P2X7$ submodel initiated from the naïve closed state $C_1$ (solid red curve), or a $P2X7$ submodel initiated from the sensitized closed state $C_4$ (dashed red curve). (B) Density distributions of experimental $Ca^{2+}$ response magnitudes to $10^{-2}$ M [ATP] in WT cells (blue density; unimodal) and $P2ry2\Delta$ cells (red density; bimodal). Vertical lines show simulated response magnitudes ($10^{-2}$ M [ATP]) obtained by the full model (solid blue line), naïve $P2X7$ submodel (solid red line) and sensitized $P2X7$ submodel (dashed red line).

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Functional contributions of P2Y2 and P2X7 to mechanotransductive signaling

To investigate the potential functional consequences of the complex interactions between P2Y2 and P2X7, we examined how the absence of each of these receptors affects ATP-mediated mechanotransduction. We have previously shown that mechanical stimulation of a single "primary" osteoblast with a glass micropipette leads to the release of $10^{-5}$ to $10^{-4}$ M ATP into the pericellular space, which then diffuses to stimulate neighbouring non-mechanically perturbed "secondary" cells [11,12]. Here, we mechanically stimulated a single fura2-loaded osteoblast from parental C2-OB, or clones deficient in P2Y2, P2ry2Δ, or P2X7, P2rx7Δ and recorded $[\text{Ca}^{2+}]_i$ responses in the primary and secondary cells (Fig 8A). We found that while in P2rx7Δ cells the response was qualitatively similar to WT, in P2ry2Δ cells secondary responses were abolished (Fig 8B and 8C). Quantitatively, the primary response was unaffected in P2ry2Δ cells, but exhibited higher magnitude and faster decay in P2rx7Δ cells (Fig 8C and 8D). The suppression of the secondary response in P2ry2Δ cells was evident by the reduced signaling radius ($p = 9 \times 10^{-3}$), fractions of responding cells ($p = 5 \times 10^{-11}$), response magnitudes ($p = 2 \times 10^{-4}$) and areas under the curves (AUC; $p = 0.02$) (Fig 8B–8D, yellow).

Fig 8. P2Y2R and P2X7R contribution to mechanically stimulated signals in bone cells. (A) Fura2-loaded C2-OB cells were plated on a glass-bottom dish and individual cell (primary; 1°) was mechanically stimulated with a glass-micropipette inducing ATP release into the extracellular space, which subsequently stimulated P2 responses in neighbouring cells (secondary; 2°). (B) Representative images of $[\text{Ca}^{2+}]_i$ (pseudocolor of 340/380 ratio) in C2-OB parental (top) and P2ry2Δ (bottom) cultures, in which a single cell was mechanically stimulated at $t = 0$ s (white arrows). The snapshot at 15 s demonstrate secondary responses in neighboring cells. Red traces: secondary responses; Black traces: primary responses; Black traces: secondary responses. (C) Time-series recordings in WT (top panel), P2ry2Δ (middle panel), and P2rx7Δ (lower panel) cells. (D) Quantification of primary and secondary $[\text{Ca}^{2+}]_i$; response parameters, including signaling radius, fractions of responding cells, response magnitudes and areas under the curves in WT (blue), P2ry2Δ (yellow), and P2rx7Δ (grey) cells. Data are means ± SEM. *p<0.05, **p<0.01 and ***p<0.001 indicate comparisons between WT and P2ry2Δ or P2rx7Δ cells assessed by ANOVA and Bonferroni-corrected t-test. AU: arbitrary units.

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contrast, in \( P2rx7 \Delta \) cells, the signaling radius and fraction of responding secondary cells was unaffected; however, the response magnitudes and areas under the curves of secondary cells were significantly higher in \( P2rx7 \Delta \) cells compared to parental C2-CB cells (Fig 8C and 8D, grey). These data demonstrate that P2Y2 receptor is critical for the secondary responses, consistent with its high sensitivity to ATP. In addition, the contribution of P2X7 to \( Ca^{2+} \) responses is evident even though extracellular ATP in these experiments remained below [ATP] required to stimulate P2X7. Taken together, these data strongly support the importance of an interplay between P2Y2R and P2X7R.

**Discussion**

In this study, we demonstrated that the patterns of P2R-mediated \( Ca^{2+} \) responses to ATP are conserved across three independent murine osteoblast models and identified the P2Y2 and P2X7 receptors as the dominant P2 receptors contributing to these responses. Based on previous experimental evidence highlighting the role of IP3 in regulating calcium responses [11], we constructed a flux-balance based mathematical model of \( Ca^{2+} \) signals induced by the activation of high affinity P2Y2R and low affinity P2X7R. Model predictions were validated by comparing the results of simulations to experimental data of ATP-generated \( Ca^{2+} \) signaling in parental and CRISPR/Cas9 -generated P2Y2 and P2Y7 knockouts in osteoblastic C2C12-BMP cells. We demonstrated that activation of P2Y2R by progressively increasing [ATP] induces a transition from transient to oscillatory to transient \( Ca^{2+} \) responses due to the biphasic nature of IP3R activation/inactivation kinetics and the interaction of SERCA pumps with IP3Rs. At high [ATP], activation of P2X7R was found to modulate the peak response magnitudes through an interplay between the biphasic nature of IP3Rs and the desensitization kinetics of P2X7Rs. Moreover, our study suggests that P2Y2 activity may also alter the kinetics of P2X7 towards favouring naive state activation. Finally, we demonstrated that functional consequences of lacking P2Y2 or P2X7 are evident beyond the absence of a signal at the expected range of ATP concentrations. Taken together, these findings support a model in which the response to ATP is not a simple superposition of individual P2 receptors, but a complex context-specific functional response build through interactions of multiple P2 receptors.

Our experimental studies focused on characterizing ATP-mediated calcium responses in osteoblasts. In investigating these calcium responses, we chose to stimulate cells with a range of physiologically-relevant ATP concentrations (10 nM to 10 mM), such that 10–100 nM ATP represented small fluxes above baseline extracellular ATP concentrations, 1–100 \( \mu \)M ATP represented concentrations achieved following mechanical stimulation, and 1–10 mM ATP represented intracellular [ATP] that has the capacity to spill into the extracellular space and stimulate P2 receptor signalling following traumatic tissue injury [3,7,11]. We have found that murine osteoblastic cells from different sources exhibit similar patterns in their ATP concentration-dependence of \( Ca^{2+} \) responses, including non-trivial features such as the transition of response from transient to oscillatory and back to transient when increasing [ATP], and the presence of two troughs in the plateau phase of the magnitude dose-response curve at high [ATP]. These finding suggest that P2 receptors contributing to these responses are also conserved. P2 receptors are ubiquitously expressed in every mammalian cell, with cell- and tissue-type specific patterns of expression [5] Consistent with previous reports [25,26], we demonstrated that the pattern of P2 receptors expressed across different murine osteoblast models is consistent at the mRNA level, and identified ATP-sensitive P2X7 and P2Y2 receptors as the dominant P2 receptor subtypes in osteoblasts. In keeping with their important roles in bone, \( P2rx7^{-/-} \) and \( P2y2^{-/-} \) mice have been shown to exhibit severe bone phenotypes, with \( P2rx7^{-/-} \) mice demonstrating significant deficiency in bone mineral density and truncated response to
mechano-adaptive loading [27,28], and P2ry2−/− mice similarly experiencing osteopenia and altered mechanotransducing responses [29]. In our study, CRISPR-Cas9 double-nickase generated clonal C2-OB cells lacking P2ry2 or P2rx7 showed altered responses to sustained ATP stimulation, which translated into significant changes in mechanotransducing [Ca^{2+}] signaling. Thus, P2Y2 and P2X7 receptors play critical roles in mediating the osteoblast response to ATP, particularly in the context of mechanotransducing signaling in bone.

In every osteoblast model, we found that there was a finite range of [ATP] over which oscillatory [Ca^{2+}] response is prevalent. The oscillatory behaviour was abolished in P2ry2Δ cells, but preserved in P2rx7Δ cells, strongly implicating P2Y2 as a mediator of oscillations. Using the mathematical model of P2Y2-induced changes in [IP₃] and [Ca^{2+}]_ER allowed us to examine the mechanism of transition between oscillatory and non-oscillatory (transient) responses. We found that moderate IP₃ production evokes an oscillatory response because of two factors: i) CICR by the IP₃Rs that exerts negative feedback on the receptors and inhibits them, and ii) the interaction of IP₃Rs with SERCA that pumps Ca^{2+} back into the ER. In contrast, at low [ATP], the Ca^{2+} released by IP₃Rs is insufficient to feedback and inhibit the receptors, whereas at high [ATP], IP₃Rs become constitutively activated, making CICR larger than that in the moderate case but no longer able to inactivate the receptors; this produces, as a result, transient Ca^{2+} responses in both cases. While this model prediction is interesting, its validation is limited by difficulties in experimentally measuring osteoblastic IP₃ dynamics. Specifically, little is known about the basal [IP₃] (assumed to be 0 μM in our model), which plays a significant role in whether solutions will pass through the oscillatory region obtained in the [IP₃] and [Ca^{2+}]_ER space. Furthermore, the ability of the model to predict some of the experimental response profiles observed at elevated [ATP] is limited by the chosen P2YR-IP₃R sub-model, which is unable to slowly decay after a rapid increase in [Ca^{2+}] [24]. Given the large heterogeneity of responses at these concentrations, choosing a simplified P2YR-IP₃R sub-model was prioritized over the ability to reproduce some response patterns. In spite of these limitations, experimental and modeling data agree on the critical role of P2Y2-induced IP₃-mediated Ca^{2+} release from ER in generating oscillatory Ca^{2+} responses to ATP.

Another conserved feature in ATP dose dependence in osteoblasts is the non-monotonic changes in the response magnitude. A similar dose-response curve, with troughs at 10^{-4} M and 10^{-2} M ATP and a peak at 10^{-3} M ATP was reported in MC3T3-E1 osteoblasts [7]. While we have previously suggested that the decrease in the response magnitude may be mediated by negative effects of one of the receptors that are active at mid-range [ATP] [7], current study demonstrates that similar regulation is achieved through interactions between P2Y2 and P2X7. In particular, we have found that these characteristic troughs disappear in P2rx7Δ cells. Using the mathematical model, we showed that at 10^{-4} M ATP, the additional Ca^{2+} influx through now activated P2X7 inhibits P2Y2-induced IP₃R activity due to the biphasic dependence of IP₃Rs on [Ca^{2+}]. As a result, the peak [Ca^{2+}] response, which is predominantly mediated by IP₃R activity, decreases, creating the first trough in the dose-response curve at 10^{-4} M [ATP]. With further increase in [ATP], the activation of P2X7Rs, known to monotonically increase with [ATP], starts to outweigh the reduced IP₃R activity, causing the peak [Ca^{2+}] response to rise, reaching a global maximum around 10^{-3} M ATP. After that, the rate of P2X7R desensitization (that increases with [ATP]) becomes large enough to impede Ca^{2+} influx through the receptors, resulting in a decrease in the peak [Ca^{2+}] response (now predominantly mediated by P2X7Rs) which creates the second trough in the dose-response curve at 10^{-2} M ATP. These data demonstrate how activation of low affinity P2X7 may affect the responses mediated by the high affinity P2Y2. Our study also suggests the reciprocal effect of P2Y2 on the function of P2X7 through facilitating the naïve state activation of P2X7. Indeed, previous studies have documented such an effect through the allosteric regulation of P2XR.
(including P2X7R) by Ca^{2+} [17,19], suggesting that Ca^{2+} release from the ER through the P2Y2 pathways may underly the altered kinetics of P2X7. Taken together, our study demonstrates multiple points of interactions between P2Y2 and P2X7 receptors, which are not only activated at very different ranges of ATP concentration, but also belong to different classes of receptors.

Finally, our study demonstrates that the absence of either P2Y2 or P2X7 has significant implications on ATP-mediated mechanotransduction. We used an experimental setup in which the mechanical stimulation of a single osteoblasts generates a micro-injury in its cell membrane, leading to a release of ATP that signals to neighboring (secondary) cells [11]. First, we showed that in the absence of P2Y2, the transmission of ATP signal to neighboring cells is effectively interrupted. These findings are consistent with previous reports that osteoblasts from P2Y2^{-/-} mice exhibited dramatic reduction in fluid flow-induced Ca^{2+} responses even though the ATP release was similar to WT osteoblasts [29]. Second, we have found that in the absence of P2X7, both primary and secondary responses are significantly altered. While local ATP concentrations at the site of micro-injury may support the involvement of P2X7 in generating the Ca^{2+} response of the primary cell [11,12], the observed changes in the secondary responses are surprising, since we have previously shown that the amount of ATP released in these experiments is below the concentrations required for P2X7 activation [11]. Nevertheless, this observation is consistent with previously suggested alterations in mechanotransductive signaling in P2X7 deficient mice [27,28]. Thus, our study supports the important role of P2 receptor network in generating a mechanotransductive signal that conveys complex information to neighboring cells.

In conclusion, this study provided a complex mechanism of interdependency between the action of high affinity G-protein coupled receptor P2Y2 and a low affinity ligand gated ion channel P2X7. Using a combination of experimental studies in osteoblastic cells with the full compliment of P2 receptors, as well as osteoblasts deficient in P2Y2 or P2X7, and mathematical modeling of P2Y2R-mediated Ca^{2+} release coupled to a Markov model of P2X7R dynamics, allowed us to explore the intricate details of the subcellular signaling induced by ATP in bone forming osteoblasts. The conclusions drawn demonstrated causative links between the exposure to mechanical force, early ATP-mediated signaling, and mechanoadaptive response of bone tissue.

Materials and methods

Software

Figure preparation: CorelDRAWX8 (Corel); Mathematical Modeling: MATLAB R2018a (MathWorks), XPPAUT 8.0. Statistical Analysis: R version 4.0.0 (R Foundation for Statistical Computing).

Solutions and reagents

Refer to S1 Text for a complete list of materials used in this study.

Cell culture

The C2C12 cell line (ATCC CRL-1772) stably transfected with BMP-2 (C2-Ob cells) was plated at 10^4 cells/cm^2 in DMEM (supplemented with 10% FBS, 1% sodium pyruvate, 1% penicillin streptomycin) and cultured for 2–3 days prior to experiments. Absence of mycoplasma contamination was verified in cryo-preserved stocks of C2-OB cells using PCR-based detection kit.
**Generation of P2R knockout cell lines**

C2-Ob cells were plated in 6-well plates at 100,000 cell/well density 2 days prior to transfection. On the day of transfection, 7.5 μL lipofectamine was diluted in 125 μL Opti-MEM medium (Solution A) and 5 μg P2ry2 or P2rx7 CRISPR/Cas9 plasmid and 10 μL P3000 reagent were diluted in a separate 125 μL aliquot of Opti-MEM (Solution B). Solutions A and B were then pooled in a 1:1 ratio and incubated at room temperature for 15 min. Cell media was aspirated and 250 μL of the pooled DNA-lipid complex solution was added to cells and left to incubate at 37˚C for 3 days. 3 days post-transfection, cells were visualized using fluorescent microscope to verify successful transfection through the presence of GFP-positive cells. Transfected cultures were transferred to fresh DMEM media and treated with 5 μM puromycin for 7 days to select for puromycin-resistant clones. After selection, cells were transferred into puromycin-free media, allowed 3 days for recovery, and re-plated in 96 well plates at a ~1 cell per well density. After 3 weeks of expansion, half of each single-cell colony was re-plated in 96-well plates and the other half was collected for genomic DNA extraction using DNeasy kit. Genomic DNA for each single-cell colony was amplified by touchdown PCR using primer sets designed to flank the genomic region targeted by Cas9 (S2 Table), and amplicons were separated on a gel to screen for clones with evident band shifts. Selected clones were subsequently validated by immunoblot analysis, and termed P2ry2Δ and P2rx7Δ cells, for P2ry2 and P2rx7, respectively.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was isolated using RNeasy kit and QIAshredder columns and reverse transcribed using cDNA reverse transcription kit. Real time qPCR was performed using QuantStudio 7 Flex PCR System, with SYBR Green or TaqMan Master Mix. Primer sequences are provided in S2 Table and cycling conditions in S3 Table.

**Intracellular Ca²⁺ recordings and analysis**

Cells were plated on glass-bottom 35 mm dishes or 48-well plates (MatTek Corporation), for single-cell mechanical stimulation and agonist application experiments, respectively. Cell were loaded with Fura2-AM for 30 min, acclimatized in physiological solution (PS) for 10 min on the stage of an inverted fluorescence microscope (Nikon T2000), and imaged as described previously [11]. For each recording, all cells within the field-of-view at 40x magnification were imaged (~15–30 cell per imaging experiment). The Ca²⁺ response parameters were analyzed using a previously developed MATLAB algorithm (https://github.com/NMikolajewicz/Calcium-Signal-Analyzer) [13]. To assess ATP dose-dependencies, Fura2-loaded C2-Ob or CB-Ob cells were bathed in 270 μL PS and 30 μL of UDPG, ATP or ADP solutions at 10x final concentration were added (e.g., 30 μL of 10⁻⁶ M ATP solution was added to cells to achieve 10⁻⁵ M ATP stimulation).

**Immunoblotting**

Cell lysates were extracted in RIPA lysis buffer and samples were prepared and subject to SDS-PAGE on a 10% (w/v) acrylamide gel as previously described [11]. Blotted nitrocellulose membranes were incubated with primary antibodies overnight (1:1000 dilution, 5% BSA in TBST, 4˚C) and secondary antibodies were applied for 1 h (1,1000 dilution, 5% BSA in TBST, rt) prior to visualization with chemiluminescence system.

**Mechanical-stimulation**

Single osteoblastic cells were stimulated by local membrane indentation with a glass micropipette using a FemtoJet microinjector NI2 (Eppendorf Inc.), as previously described [11].
**Statistical analysis**

Data are presented as representative images, means ± standard error (SE) or means ± 95% confidence intervals (CI), as specified in each figure panel along with sample sizes \( N \) (number of independent experiments) and \( n \) (number of technical replicates). Curve fitting and \([\text{Ca}^{2+}]_i\) transient characterization were performed in R. Statistical significance was assessed by one- or two-way ANOVA (as specified) and post-hoc two-way unpaired Students’ t-tests were adjusted using the Bonferroni correction. Significance levels were reported as single symbol (\( *p < 0.05 \)), double symbol (\( **p < 0.01 \)) or triple symbol (\( ***p < 0.001 \)).

**Mathematical model**

The mathematical model consisted mainly of Eqs (1)–(3). The individual terms \( J_{\text{INleak}}, J_{\text{IPR}}, J_{\text{ERleak}}, J_{\text{PMCA}}, J_{\text{SERCA}} \) and \( J_{\text{P}2\text{X}7} \), listed in Eqs (1) and (2), were the key \( \text{Ca}^{2+} \) fluxes considered in this model, as described below.

(1) **Plasma Membrane Ca\(^{2+}\) Leak** \( (J_{\text{INleak}}) \). A constant inward leak across cell membrane to ensure that total \([\text{Ca}^{2+}] \) within the cell remained positive. It was assumed to be constant (see Table 1).

(2) **IP\(_3\)R Ca\(^{2+}\) Flux** \( (J_{\text{IPR}}) \): The \( \text{Ca}^{2+} \) flux through IP\(_3\)Rs, given by

\[
J_{\text{IPR}} = v_{\text{IPR}} O_{\text{IPR}} ([\text{Ca}^{2+}]_{\text{ER}} - [\text{Ca}^{2+}]_i),
\]

where \( v_{\text{IPR}} \) is the maximum rate of \( \text{Ca}^{2+} \) release by the IP\(_3\)R and \( O_{\text{IPR}} \) is the IP\(_3\)R open probability, defined by

\[
O_{\text{IPR}} = m_\infty n_\infty h_\infty,
\]

which follows a Hodgkin-Huxley gating formalism adopted by De Young and Keizer [30] and later simplified by Li and Rinzel [24]. In this simplification, the activation by IP\(_3\)R (defined by \( m_\infty \)) and \([\text{Ca}^{2+}]_i\) (defined by \( n_\infty \)) through binding to the receptor were assumed to be instantaneous, given by

\[
m_\infty = \frac{[\text{IP}_3]}{[\text{IP}_3] + d1} \quad \text{and} \quad n_\infty = \frac{[\text{Ca}^{2+}]_i}{[\text{Ca}^{2+}]_i + d3}
\]

whereas the inactivation by \([\text{Ca}^{2+}]_i\) (defined by the gating variable \( h \)), also through binding, was assumed to occur at a much slower time scale governed dynamically by

\[
\frac{dh}{dt} = \frac{h_\infty - h}{\tau_h},
\]

where

\[
\tau_h = \frac{1}{a_s(Q_2 + [\text{Ca}^{2+}])}, \quad h_\infty = \frac{Q_2}{Q_2 + [\text{Ca}^{2+}]}, \quad \text{and} \quad Q_2 = d1 \frac{[\text{IP}_3]}{[\text{IP}_3] + d1}.
\]

In the study by De Young and Keizer [30] the values of \( d_i \) \( (i = 1, 2, 3, 5) \) were fit to experimental data [31]. Note that the dependence of activation and inactivation of \( O_{\text{IPR}} \) on \([\text{Ca}^{2+}]_i\) in Eq (5) due to CICR makes the profile of IP\(_3\)R open probability biphasic with respect to \([\text{Ca}^{2+}]_i\).  

(3) **ER Ca\(^{2+}\) Leak** \( (J_{\text{ERleak}}) \). A small leak across the ER membrane [24], given by

\[
J_{\text{ERleak}} = v_{\text{ERleak}} ([\text{Ca}^{2+}]_{\text{ER}} - [\text{Ca}^{2+}]_i),
\]

where \( v_{\text{ERleak}} \) is the maximal rate of \( \text{Ca}^{2+} \) leak from the ER.
(4) Ca$^{2+}$ ATPase Activity ($I_{\text{PMCA}}$ and $I_{\text{SERCA}}$). Ca$^{2+}$ removal by PMCA and Ca$^{2+}$ re-uptake into the ER by SERCA described by Hill functions [32,33], given by

$$I_x = v_x \frac{[\text{Ca}^{2+}]^{n_x}}{[\text{Ca}^{2+}]^{n_x} + k_x^2},$$

where $x = \text{PMCA, SERCA}$, $v_x$ is the maximal pumping rate and $k_x$ is the affinity of the pump to bind to Ca$^{2+}$.

(5) P2X7R Ca$^{2+}$ Flux ($I_{\text{P2X7}}$). Ca$^{2+}$ flux through P2X7Rs. A 12-state Markov model [18] was initially used to compute the current, given by

$$I_{\text{P2X7}} = g_{\text{X7}} (Q_1 + Q_2 + Q_3 + Q_4) (V - E),$$

where $g_{\text{X7}}$ is the conductance of the P2X7R open states $Q_i$, $i = 1$–$4$ [18]. With emerging evidence suggesting that P2XRs do not dilate [16,34], the maximum conductance of open ($Q_1$ and $Q_2$) and sensitized/primed ($Q_3$ and $Q_4$) states in this P2X7R sub-model were assumed to be equal. We also assumed that the rate of desensitization increased with ATP binding ($H_2(C_2) < H_2(Q_1) < H_2(Q_2)$) and that the open probability is higher in the desensitized row (i.e., $k_2 > k_j$, $j = 2, 4, 6$). These modifications kept the time series simulations of the P2X7R model generally unchanged. To obtain the overall Ca$^{2+}$ flux through these channels, we then used the formalism from Zeng et al. [35] to convert ionic current to flux, scaled by a fraction that represents the average Ca$^{2+}$ flux [36] relative to that for Na$^+$ and K$^+$. The latter was necessary as P2X7Rs are non-selective cation channels. Using the above description, the following expression was used to describe this flux

$$I_{\text{P2X7}} = \frac{f_{\text{Ca}} I_{\text{P2X7}}}{2FV_{\text{oste}}},$$

where $z$ is the valence of Ca$^{2+}$ ($z = +2$), $F$ is Faraday’s constant, $V_{\text{oste}}$ is the volume of the osteoblast in liters and $f_{\text{Ca}}$ is the fraction of Ca$^{2+}$ flux through P2X7R.

**Slow-fast analysis**

“Slow-fast” analysis is a mathematical technique that divides a dynamics model, such as the one described by Eqs (1)–(12), into two subsystems: a fast and slow one, followed by setting the slow variables to be independent parameters. The goal in doing so is to analyze the dynamics of the fast subsystem with respect to those newly defined parameters. This well-known technique that was also applied in [24] allows you to define the distinct regions of behaviour with respect to the slow variables and determine how trajectories of the original model could evolve in time when passing between these regions.

**Software and numerical methods**

All time-series simulations were performed in MATLAB (Mathworks, Natick, MA). Initially, all simulations were run for a period of 2000 s in the absence of ATP to obtain the resting state of the cell. The basal [IP$_3$] (the [IP$_3$] in the absence of extracellular ATP) was assumed to be zero. P2Y2R and P2X7R knockout recordings were simulated by setting $I_{\text{P2X7}}$ and $I_{\text{IP3R}}$ (Eqs (5) and (12)) to zero, respectively. These simulations were then used to compute [ATP]-dependent dose-response curves of [Ca$^{2+}$], by evaluating the maximum Ca$^{2+}$ response at each ATP dose in MATLAB. The bifurcation analysis (Fig 5) was performed using XPPAUT (a free-ware available online at [http://www.math.pitt.edu/~bard/xpp/xpp.html](http://www.math.pitt.edu/~bard/xpp/xpp.html)). To facilitate reproduction of results, the codes used to perform simulations of the model can be obtained online.
These simulations can be run by solving the function “fullmodel.m” using the ordinary differential equation solver ode15s. Figs 4A, 6 and 7 can be obtained by running the files titled “Fig 4A.m”, “Fig 6.m” and “Fig 7.m”, respectively.

Supporting information

S1 Fig. Aligning and scaling ATP dose-dependence curves. (A-C) Schematic illustrating processing of ATP-dose-dependent response curves (A). ATP dose-dependent responses from three independent murine cell lines (A, left panel; B) were aligned using a linear transformation to match peaks/troughs (A, middle panel; C) and responses were rescaled to [0,1] interval (A, right panel; Fig 1C). Curves: Loess curves; Markers: Response means (circle: BM-OB; triangle: C2-OB; square: CB-OB).

S1 Table. Calcium response parameters.

S2 Table. Primer and PAM sequences.

S3 Table. PCR cycling conditions.

S1 Text. Solutions and Reagents.

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References

1. Verkhratsky A, Burnstock G. Biology of purinergic signalling: its ancient evolutionary roots, its omnipresence and its multiple functional significance. Bioessays. 2014; 36(7):697–705. https://doi.org/10.1002/bies.201400024 PMID: 24782352

2. Burnstock G, Ralevic V. Purinergic signaling and blood vessels in health and disease. Pharmacological reviews. 2014; 66(1):102–92. https://doi.org/10.1124/pr.113.008209 PMID: 24335194

3. Mikolajewicz N, Mohammed A, Morris M, Komarova SV. Mechanically stimulated ATP release from mammalian cells: systematic review and meta-analysis. Journal of Cell Science. 2018; 131(22). https://doi.org/10.1242/jcs.223354 PMID: 21622280

4. Bours M, Dagnelie PC, Giuliani AL, Wesselius A, Di Virgilio F. P2 receptors and extracellular ATP: a novel homeostatic pathway in inflammation. Front Biosci (Schol Ed). 2011; 3:1443–56. https://doi.org/10.2741/235 PMID: 21622280

5. Burnstock G. Physiology and pathophysiology of purinergic neurotransmission. Physiological reviews. 2007. https://doi.org/10.1152/physrev.00043.2006 PMID: 17429044

6. Jacobson KA, Costanzi S, Joshi BV, Besada P, Shin DH, Ko H, et al., editors. Agonists and antagonists for P2 receptors. Novartis Foundation symposium; 2006: Wiley Online Library.

7. Xing S, Grol MW, Gutter PH, Dixon SJ, Komarova SV. Modeling interactions among individual P2 receptors to explain complex response patterns over a wide range of ATP concentrations. Frontiers in physiology. 2016; 7:294. https://doi.org/10.3389/fphys.2016.00294 PMID: 27468270

8. Coddou C, Yan Z, Obsil T, Huidobro-Toro JP, Stojilkovic SS. Activation and regulation of purinergic P2X receptor channels. Pharmacological reviews. 2011; 63(3):641–83. https://doi.org/10.1124/pr.110.003129 PMID: 21737531

9. Erb L, Weisman GA. Coupling of P2Y receptors to G proteins and other signaling pathways. Wiley Interdisciplinary Reviews: Membrane Transport and Signaling. 2012; 1(6):789–803. https://doi.org/10.1002/wmta.62 PMID: 23749333

10. Grol MW, Pereverzev A, Sims SM, Dixon SJ. P2 receptor networks regulate signaling duration over a wide dynamic range of ATP concentrations. J Cell Sci. 2013; 126(Pt 16):3615–26. https://doi.org/10.1242/jcs.122705 PMID: 23750003

11. Mikolajewicz N, Zimmermann EA, Willie BM, Komarova SV. Mechanically stimulated ATP release from murine bone cells is regulated by a balance of injury and repair. Elife. 2018; 7:e37812. https://doi.org/10.7554/eLife.37812 PMID: 30324907

12. Mikolajewicz N, Sehayek S, Wiseman PW, Komarova SV. Transmission of mechanical information by purinergic signaling. Biophysical journal. 2019; 116(10):2039–52. https://doi.org/10.1016/j.bpj.2019.04.012 PMID: 31053281

13. Mackay L, Mikolajewicz N, Komarova SV, Khadra A. Systematic characterization of dynamic parameters of intracellular calcium signals. Frontiers in physiology. 2016; 7:525. https://doi.org/10.3389/fphys.2016.00525 PMID: 27891096

14. North RA. Molecular physiology of P2X receptors. Physiological reviews. 2002. https://doi.org/10.1152/physrev.00015.2002 PMID: 12270951

15. Burnstock G. Purine and purinergic receptors. Brain and Neuroscience Advances. 2018; 2:2398212818817494. https://doi.org/10.1177/2398212818817494 PMID: 32166165

16. Mackay L, Zemkova H, Stojilkovic SS, Sherman A, Khadra A. Deciphering the regulation of P2X4 receptor channel gating by ivermectin using Markov models. PLoS computational biology. 2017; 13(7):e1005643. https://doi.org/10.1371/journal.pcbi.1005643 PMID: 28708827

17. Khadra A, Yan Z, Coddou C, Tomić M, Sherman A, Stojilkovic SS. Gating properties of the P2X2a and P2X2b receptor channels: experiments and mathematical modeling. J Gen Physiol. 2012; 139(5):333–48. https://doi.org/10.1085/jgp.201110716 PMID: 22547664

18. Khadra A, Tomić M, Yan Z, Zemkova H, Sherman A, Stojilkovic SS. Dual gating mechanism and function of P2X7 receptor channels. Biophys J. 2013; 104(12):2612–21. https://doi.org/10.1016/j.bpj.2013.05.006 PMID: 23790369

19. Yan Z, Khadra A, Sherman A, Stojilkovic SS. Calcium-dependent block of P2X7 receptor channel function is allosteric. J Gen Physiol. 2011; 138(4):437–52. https://doi.org/10.1085/jgp.201110647 PMID: 21911484
20. Yan Z, Khadra A, Li S, Tomic M, Sherman A, Stojilkovic SS. Experimental characterization and mathematical modeling of P2X7 receptor channel gating. J Neurosci. 2010; 30(42):14213–24. https://doi.org/10.1523/JNEUROSCI.2390-10.2010 PMID: 20962242

21. Zemkova H, Khadra A, Rokic MB, Tvrdonova V, Sherman A, Stojilkovic SS. Allosteric regulation of the P2X4 receptor channel pore dilation. Pflugers Arch. 2015; 467(4):713–26. https://doi.org/10.1007/s00424-014-1546-7 PMID: 24917516

22. Lemon G, Brockhausen J, Li GH, Gibson WG, Bennett MR. Calcium mobilization and spontaneous transient outward current characteristics upon agonist activation of P2Y2 receptors in smooth muscle cells. Biophys J. 2005; 88(3):1507–23. https://doi.org/10.1529/biophysj.104.043976 PMID: 15556987

23. Fedorov IV, Rogachevskaja OA, Kolesnikov SS. Modeling P2Y receptor-Ca2+ response coupling in taste cells. Biochim Biophys Acta. 2007; 1768(7):1727–40. https://doi.org/10.1016/j.bbamem.2007.04.002 PMID: 17512897

24. Li Y, Rinzel J. Equations for InsP3 receptor-mediated [Ca2+]i oscillations derived from a detailed kinetic model: a Hodgkin-Huxley like formalism. Journal of theoretical Biology. 1994; 166(4):461–73. https://doi.org/10.1006/jtbi.1994.1041 PMID: 8176949

25. Hoebertz A, Townsend-Nicholson A, Glass R, Burnstock G, Arnett T. Expression of P2 receptors in bone and cultured bone cells. Bone. 2000; 27(4):503–10. https://doi.org/10.1016/s8756-3282(00)00351-3 PMID: 11033445

26. Orriss IR, Key ML, Brandao-Burch A, Patel JJ, Burnstock G, Arnett TR. The regulation of osteoblast function and bone mineralisation by extracellular nucleotides: The role of p2x receptors. Bone. 2012; 51 (3):389–400. https://doi.org/10.1016/j.bone.2012.06.013 PMID: 22749899

27. Ke HZ, Qi H, Weidema AF, Zhang Q, Panupinthu N, Crawford DT, et al. Deletion of the P2X7 nucleotide receptor reveals its regulatory roles in bone formation and resorption. Molecular Endocrinology. 2003; 17(7):1356–67. https://doi.org/10.1210/me.2003-0027 PMID: 12677101

28. Zeng D, Yao P, Zhao H, P2X7, a critical regulator and potential target for bone and joint diseases. Journal of cellular physiology. 2019; 234(3):2095–103. https://doi.org/10.1002/jcp.27544 PMID: 30317588

29. Xing Y, Gu Y, Bresnahan JJ, Paul EM, Donahue HJ, You J. The roles of P2Y2 purinergic receptors in osteoblasts and mechanotransduction. PLoS One. 2014; 9(9):e108417. https://doi.org/10.1371/journal.pone.0108417 PMID: 25268784

30. De Young GW, Keizer J. A single-pool inositol 1, 4, 5-trisphosphate-receptor-based model for agonist-stimulated oscillations in Ca2+ concentration. Proceedings of the National Academy of Sciences. 1992; 89(20):9895–9. https://doi.org/10.1073/pnas.89.20.9895 PMID: 13291089

31. Watras J, Ehrlich BE. Bell-shaped calcium-response curves of Ins (l, 4, 5) P 3-and calcium-gated channels from endoplasmic reticulum of cerebellum. Nature. 1991; 351(6329):751–4. https://doi.org/10.1038/351751a0 PMID: 1648178

32. Croisier H, Tan X, Perez-Zoghbi JF, Sanderson MJ, Sneyd J, Brook BS. Activation of store-operated calcium entry in airway smooth muscle cells: insight from a mathematical model. PloS one. 2013; 8(7):e69598. https://doi.org/10.1371/journal.pone.0069598 PMID: 23936056

33. Chen Y-I, Cao J, Zhong J-n, Chen X, Cheng M, Yang J, et al. Plasma membrane Ca2+-ATPase regulates Ca2+ signaling and the proliferation of airway smooth muscle cells. European Journal of Pharmacology. 2014; 740:733–41. https://doi.org/10.1016/j.ejphar.2014.05.055 PMID: 24912144

34. Li M, Toombes GE, Silberberg SD, Swartz KJ. Physical basis of apparent pore dilation of ATP-activated P2X receptor channels. Nature neuroscience. 2015; 18(11):1577–83. https://doi.org/10.1038/nn.4120 PMID: 26389841

35. Zeng S, Li B, Zeng S, Chen S. Simulation of spontaneous Ca2+ oscillations in astrocytes mediated by voltage-gated calcium channels. Biophysical journal. 2009; 97(9):2429–37. https://doi.org/10.1016/j.bpj.2009.08.030 PMID: 19883685

36. Egan TM, Khakh BS. Contribution of calcium ions to P2X channel responses. Journal of Neuroscience. 2004; 24(13):3413–20. https://doi.org/10.1523/JNEUROSCI.5429-03.2004 PMID: 15056721

37. Mikolajewicz N, Smith D, Komarova SV, Khadra A. High-affinity P2Y2 and low-affinity P2X7 receptor interaction modulates ATP-mediated calcium signalling in murine osteoblasts. Annar Khadra Repository 2020 2021:Available from: www.medicine.mcgill.ca/physio/khadralab/Codes/code_ploscomp2.html

38. Tsai J-C, Zhang W, Kirk V, Sneyd J. Traveling waves in a simplified model of calcium dynamics. SIAM Journal on Applied Dynamical Systems. 2012; 11(4):1149–99.

39. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. The endoplasmic reticulum. Molecular Biology of the Cell 4th edition: Garland Science; 2002.
40. Zouani OF, Rami L, Lei Y, Durrieu M-C. Insights into the osteoblast precursor differentiation towards mature osteoblasts induced by continuous BMP-2 signaling. Biology open. 2013; 2(9):872–81. https://doi.org/10.1242/bio.20134986 PMID: 24143273

41. Matsu-ura T, Michikawa T, Inoue T, Miyawaki A, Yoshida M, Mikoshiba K. Cytosolic inositol 1, 4, 5-trisphosphate dynamics during intracellular calcium oscillations in living cells. Journal of Cell Biology. 2006; 173(5):755–65. https://doi.org/10.1083/jcb.200512141 PMID: 16754959