Mechanically-stimulated ATP release from murine bone cells is regulated by a balance of injury and repair

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Impact statement:

In vitro and in vivo mechanical stimulation experiments reveal a mechano-adaptive mechanism of ATP release from bone cells, in which the amount of ATP released is initially proportional to mechanically induced non-lethal membrane injury and is later limited by adaptive membrane repair through PKC-dependent vesicular exocytosis.
Abstract

Bone cells sense and actively adapt to physical perturbations to prevent critical damage. ATP release is among the earliest cellular responses to mechanical stimulation. Mechanical stimulation of a single murine osteoblast led to the release of 70 ± 24 amole ATP, which stimulated calcium responses in neighboring cells. Osteoblasts contained ATP-rich vesicles that were released upon mechanical stimulation. Surprisingly, interventions that promoted vesicular release reduced ATP release, while inhibitors of vesicular release potentiated ATP release. Searching for an alternative ATP release route, we found that mechanical stresses induced reversible cell membrane injury in vitro and in vivo. Ca$^{2+}$/PLC/PKC-dependent vesicular exocytosis facilitated membrane repair, thereby minimizing cell injury and reducing ATP release. Priming cellular repair machinery prior to mechanical stimulation reduced subsequent membrane injury and ATP release, linking cellular mechanosensitivity to prior mechanical exposure. Thus, our findings position ATP release as an integrated readout of membrane injury and repair.
Introduction

The mechanical environment is an important determinant of bone health, as emphasized by a consistent bone loss in astronauts exposed to microgravity or in paralyzed or bedridden patients (1). Gravitational and muscle forces act on the skeleton during physical activity resulting in a complex combination of shear forces, strains and pressures. Bone-embedded osteocytes and bone-forming osteoblasts are widely regarded as the mechanosensitive cells in the skeletal system (2).

Following mechanical stimulation of rodent and human osteoblasts, transient intracellular free calcium ([Ca$^{2+}$]$_i$) elevations and ATP release are among the earliest detectable events, which result in autocrine and paracrine purinergic (P2) receptor signaling (3-5). Vesicular release of lysosomes or secretory vesicles, or conductive release via channels such as Maxi anion channels, Volume-regulated anion channels, Connexins or Pannexins are the main mechanisms of regulated ATP release in mammalian cells (6). Pathological ATP spillage also occurs from traumatically damaged cells (6). Of interest, non-lethal membrane injury has been demonstrated in vivo in several tissues (7), including bone (8), under physiological conditions. The mechanism of facilitated cell membrane repair has been described and involves Ca$^{2+}$/PKC-dependent vesicular exocytosis (9). However, the contribution of non-lethal cell injury to ATP release and related mechanotransductive purinergic signaling remains unclear.

The goal of this study was to examine the mechanism of ATP release from mechanically-stimulated cells of the osteoblastic lineage. Since we have previously demonstrated that transient membrane disruption is required to induce global [Ca$^{2+}$]$_i$ elevations in osteoblasts (10), we were particularly interested in understanding the contribution of membrane injury to mechanically-induced ATP release. Mechanical forces were applied by local membrane deformation or turbulent fluid shear stress in vitro to BMP-2 transfected C2C12 osteoblastic cells (C2-OB), primary bone marrow (BM-OB) and compact bone (CB-OB)-derived osteoblasts and changes in [Ca$^{2+}$]$_i$, vesicular exocytosis, membrane permeability and ATP release were assessed. The prevalence of membrane injury in osteocytes at physiological and supraphysiological mechanical strain levels was investigated following in vivo cyclic compressive tibial loading of 10-week old female C57Bl/6J mice.

Results

Mechanically-stimulated osteoblasts release ATP that induces calcium responses in non-stimulated neighboring cells

Osteoblasts from three different sources, C2-OB, CB-OB, and BM-OB, were loaded with [Ca$^{2+}$]$_i$ dye Fura2 and mechanically stimulated with a glass micropipette, which induced qualitatively similar transient global [Ca$^{2+}$]$_i$ elevations, consistent with prior work (3-5) (Fig. 1A-C, Fig. 1-
video 1). L-type voltage-sensitive calcium channel (VSCC) inhibitor Nifedipine and P2 antagonist PPADS significantly reduced the amplitude of mechanically-stimulated [Ca\textsuperscript{2+}]\textsubscript{i} transients (Fig. 1D). L-type VSCC activation occurred gradually (Fig. 1E) while the P2 receptor-driven component of the response peaked within seconds of stimulation (Fig. 1F). Together, L-type VSCC and P2 receptor-driven component accounted for ~50% of the mechanical stimulated [Ca\textsuperscript{2+}]\textsubscript{i} transient. Consistent with previous reports (3-5), shortly after a single osteoblast was mechanically stimulated, neighboring cells exhibited delayed secondary [Ca\textsuperscript{2+}]\textsubscript{i} responses (Fig. 1G). Pharmacological interventions revealed that P2 receptors mediated the secondary response in all three osteoblast models, while a tendency for GAP junction involvement was observed in BM-OB responses (Fig. 1H). Puff application of 10 µM ATP mimicked the appearance of secondary responders in C2-OB (Fig. 1I).

We used a luciferin fluorescence-based imaging technique (11) to directly measure ATP release ([ATP]\textsubscript{e}) (Fig. 2A, Fig. S1). Simultaneous recording of [ATP]\textsubscript{e} and [Ca\textsuperscript{2+}]\textsubscript{i} demonstrated that ATP release occurred within seconds after mechanical stimulation (time to peak release: 1.64 ± 0.36 s) and preceded the onset of [Ca\textsuperscript{2+}]\textsubscript{i} responses in neighboring cells (Fig. 2). We measured pericellular [ATP]\textsubscript{e} concentrations in the range of 0.05 – 80.5 µM corresponding to a release of 70 ± 24 amole ATP from a single mechanically stimulated osteoblast (Fig. 2B). Pericellular [ATP]\textsubscript{e} significantly correlated with the percentage of secondary responders (Fig. 2C).

**Vesicular ATP released upon mechanical stimulation is not the primary source of extracellular ATP**

Vesicular ATP release was proposed to be a major source of extracellular ATP (5). Using confocal microscopy, we found that an acidophilic marker quinacrine and a fluorescent ATP analog MANT-ATP co-localized in intracellular granular compartments (Fig. 3A, Fig. 3-videos 1-3). Quinacrine-positive vesicular pool was entirely released after treatment with Ca\textsuperscript{2+} ionophore ionomycin, and only partially by mechanical stimulation (Fig. 3-videos 3-6), while negligible vesicular release was observed in unstimulated cells (Fig. 3B). Interestingly, vesicular release peaked within 20 s of mechanical stimulation (Fig. 3C, D), while ATP release within ~2 s (Fig. 2A). Basal vesicular density was 70 ± 4 × 10\textsuperscript{3} vesicles/µm\textsuperscript{2} (Fig. 3E) and mechanical stimulation resulted in the cumulative release of 5 ± 1 × 10\textsuperscript{3} vesicles/µm\textsuperscript{2} over 100 s post-stimulation (7.2 ± 1.2% of the basal vesicular pool) (Fig. 3F). In [Ca\textsuperscript{2+}]\textsubscript{c}-free conditions vesicular density was unaffected, while mechanically-evoked vesicular release was markedly suppressed (Fig. 3E, F). Treatment with N-ethylmaleimide (NEM) caused a 33 ± 2% increase in the vesicular density (Fig. 3E), consistent with a NEM-induced increase in the readily releasable vesicular pool (12), and a 55 ± 8% increase in mechanically-evoked vesicular exocytosis (Fig. 3F). Surprisingly, the percentage of secondary responders and activation rates for Ca\textsuperscript{2+} responses (response amplitude divided by time for signal to increase from 10 to 90% of peak) were unaffected in [Ca\textsuperscript{2+}]\textsubscript{c}-free conditions, while NEM induced significant decline in the percentage of
secondary responders and activation rates (Fig. 3G, H). We measured bulk ATP release following mechanical stimulation by media displacement, a model for turbulent fluid shear stress (tFSS) (Fig. 3I). Proportionally to the extent of tFSS, 21 ± 11 to 422 ± 97 amol ATP/cell were released, approaching 31.5% of total intracellular ATP at high tFSS (Fig. 3J). tFSS (10x media displacements) induced release of 324 ± 50 amol ATP/cell, while ionomycin only led to release of 94 ± 5 amol ATP/cell. Moreover, tFSS-induced ATP release was significantly potentiated under [Ca$^{2+}$]_i-free conditions (961 ± 88 amol ATP/cells) and inhibited in NEM-treated cells (41 ± 24 amol ATP/cells) (Fig. 3K). We also investigated conductive channels, however pharmacological inhibition of Maxi-anion channels, P2X7, Connexins, Pannexins, T-type VSCC, TRPV4 or Piezo1 channels did not affect tFSS-stimulated ATP release (Fig. 3-filg. S1). Although inhibition of L-Type VSCC had a small inhibitory effect (Fig. 3-filg. S1), our data suggest that conductive channels cannot account for ATP release observed from mechanically-stimulated osteoblasts. Importantly, our data show that increases in vesicular release were consistently associated with a reduction in mechanically-stimulated ATP release, while inhibition of vesicular exocytosis increased ATP release from mechanically-stimulated osteoblasts.

Mechanical stimuli regularly and reversibly compromise the integrity of the osteoblast membrane

We hypothesized that mechanically-stimulated ATP release is related to membrane injury. Adopting a Fura2-based dye-leakage assay (9), we examined changes in 340 ex/510 em Fura2 fluorescence following mechanical stimulation of osteoblasts (Fig. 4A). In ~40% of micropipette-stimulated osteoblasts, intracellular Fura2 fluorescence returns to pre-stimulation basal (minor or no injury; mIn). However, in 30-40% of osteoblasts post-stimulation fluorescence was partially reduced (intermediate injury; iIn) and in 15-20% of osteoblasts completely lost (severe injury; sIn) (Fig. 4B). The extent of injury sustained by a micropipette-stimulated cell correlated with the magnitude and duration of [Ca$^{2+}$]_i elevations (Fig. 4C-E), as well as the percentage of secondary responders (Fig. 4F), suggesting that ATP release is related to the extent of membrane disruption. In a dye uptake assay, we added membrane impermeable dyes, trypan blue (TB) or rhodamine-conjugate dextran (R-dextran), to osteoblasts before or after mechanical stimulation (Fig. 4G). When the dyes were applied prior to tFSS, the proportion of dye-permeable osteoblasts positively correlated with the degree of tFSS. The extent of dye uptake was higher for TB (~900 Da) compared to R-dextran (~10 kDa) (Fig. 4H, I). However, when membrane permeability was examined 300 s after stimulation, a significantly lower percentage of cells were permeable to the dyes (Fig. 4H, I). Hemichannel involvement was excluded, since hemichannel blockers did not affect mechanically-stimulated [Ca$^{2+}$]_i elevations, membrane permeability or ATP release (Fig. 4-filg. S1). Consistent with transient membrane disruption, lactate dehydrogenase (LDH, ~140 kDa) leaked into the extracellular media proportionally to tFSS magnitude (Fig. 4J), even though cell viability was minimally affected 1
After tFSS stimulation (Fig. 4K). Examining TB uptake following micropipette stimulation, we have found that all C2-OB were TB-permeable immediately upon stimulation, decreasing to 75 ± 22% within 10 s, to 40-50% by 180 s and to 18 ± 12% by 300 s (Fig. 4L). Comparing these data to dye leakage experiments suggested that minor injuries resealed within 10 s, intermediate – within 20-180 s and severe injuries did not reseal by 300 s (Fig. 4L). Based on predicted molecular radii of the dyes (14), we estimated the membrane lesion radius to be on the nanometer scale, sufficiently large to permit the efflux of smaller ATP molecules (~507 Da) (Fig. 4M).

**Osteocyte membranes are reversibly disrupted during mouse *in vivo* tibia loading**

To determine whether mechanically-induced repairable membrane disruptions occur *in vivo*, 10-week-old female C57Bl/6J mice were injected with lysine-fixable Texas Red-conjugated dextran (LFTR-Dex, ~10 kDa) either 30 min prior to or 20 min after cyclic compressive tibial loading to strain magnitudes at the upper level of physiological activities (600 µε) or at supraphysiological osteogenic levels (1200 µε) (15) (Fig. 5A-D, Fig. 5-video 1). In the contralateral non-loaded control tibiae (which experienced habitual loads only), sclerostin-positive osteocytes were observed at a density of 3212 ± 287 cells/mm² (Fig. 5-figure S1), ~4-7% of which demonstrated LFTR-Dex uptake, while only 2% of calvarial osteocytes were LFTR-Dex positive (Fig. 5E). Cyclic compressive tibial loading in the presence of LFTR-Dex resulted in significantly increased osteocyte dye uptake compared to unloaded bone, demonstrating that *in vivo* mechanical loading results in cellular membrane disruption (Fig. 5F, G red). Importantly, when LFTR-Dex was administered 20-50 min after 600 µε loading, the levels of cellular dye uptakes were similar to those in unloaded tibia, suggesting that damage evident immediately after loading was repaired within this time period (Fig. 5F light blue). Of interest, when LFTR-Dex was administered 20-50 min after 1200 µε loading, the proportion of osteocyte exhibiting dye uptake was significantly lower compared to non-loaded controls, suggesting that adaptive improvements in membrane integrity occurred in response to the supraphysiological strain (Fig. 5F, G dark blue).

**PKC regulates vesicular exocytosis and membrane resealing**

Vesicular exocytosis has been demonstrated to facilitate membrane repair and improve membrane integrity in a PKC dependent manner (9). We examined the role of Ca²⁺/PLC/PKC pathway during mechanically-induced membrane injury and resealing. Pharmacological activation of PKC (Fig. 6A, Fig. 6-figure S1A) and NEM-induced potentiation of vesicular release (Fig. 6-figure S2A) both attenuated tFSS-induced membrane disruption. Depletion of [Ca²⁺]ₘ or pharmacological inhibition of PLC (Fig. 6-figure S2A, B) or PKC (Fig. 6A, B, Fig. 6-figure S1A, B) increased membrane disruption in osteoblasts following tFSS or micropipette stimulation. Together these findings suggest that membrane repair was regulated by extracellular calcium.
influx, PLC/PKC signalling and vesicular exocytosis. We next investigated the link between Ca\(^{2+}\)/PLC/PKC pathway and mechanically-stimulated [Ca\(^{2+}\)]\(_e\) elevations, vesicular exocytosis and ATP release. Depletion of [Ca\(^{2+}\)]\(_e\) or PLC inhibition consistently reduced the amplitude of mechanically-stimulated [Ca\(^{2+}\)]\(_e\) elevations (Fig. 6-fig. S2C) and vesicular release (Fig. 6-fig. S2D), and potentiated ATP release (Fig. 6-fig. S2E). PKC activation had minor effect on calcium responses (Fig. 6-fig. S2C), augmented vesicular release (Fig. 6C) and reduced ATP release (Fig. 6D, Fig. 6-fig. S1C). In contrast, PKC inhibition potentiated calcium responses (Fig 6-fig S2C), suppressed vesicular release (Fig. 6C) and increased ATP release (Fig. 6D, Fig. 6-fig. S1C). PMA-induced PKC activation rescued vesicle pool depletion following PLC inhibition, thereby positioning PKC signalling downstream of PLC (Fig. 6-fig. S3). Together these data suggest that mechanically-stimulated membrane injury and repair, vesicular release and ATP release are regulated by Ca\(^{2+}\)/PLC/PKC-dependent signalling.

We next examined which PKC isoform is involved in the mechanoresponse. Immunoblot analysis demonstrated that mechanical stimulation (using tFSS) of CB-OB activated conventional PKCa/β and PKD/PKC\(\mu\), but not atypical PKCζ/λ or novel PKCδ/θ isoforms (Fig. 6E, Fig. 6-fig. S4). PMA stimulated phosphorylation of conventional and PKD/PKC\(\mu\) isoforms, but not atypical or novel PKC isoforms, while only basal PKD/PKC\(\mu\) phosphorylation had a tendency (p=0.09) to be inhibited by Bis. Total PKC levels were unaffected by pharmacological interventions or mechanical stimulation (Fig. 6-fig. S4G). These findings suggest that the PKD/PKC\(\mu\) isoform regulates membrane resealing in osteoblasts.

To establish the relationship between vesicular release, membrane disruption and ATP release, we pooled data for the mechanically-induced responses from all treatments studied in CB-OB (Fig. 6F-H). Mechanically-induced vesicular release negatively correlated with the extent of membrane disruption (Fig. 6F) and the amount of ATP released (Fig. 6G), while membrane disruption positively correlated with amount of ATP released in response to mechanical stimulation (Fig. 6H). These data support a model in which mechanical disruption of the cell membrane leads to ATP spillage, while the extent of membrane resealing, regulated by Ca\(^{2+}\)/PLC/PKC-dependent vesicular exocytosis, limits the amount of total ATP released (Fig. 7).

Discussion

This study establishes a novel mechanism of mechanotransduction in bone cells, which positions ATP release as the integrated outcome of membrane injury (reflective of the destructive potential of mechanical forces) and membrane repair (countering and adapting to the damage). We report that mechanical stimuli in vitro and in vivo induced reversible cell membrane disruption. This disruption resulted in ATP release predominantly through the compromised membrane, rather than through vesicular or conductive mechanisms. Ca\(^{2+}\)/PLC/PKC-dependent vesicular release was necessary for successful membrane repair and prevention of further ATP release.
Importantly, prior activation of PKD/PKCµ or vesicular priming improved membrane integrity and repair, thereby minimizing membrane injury and ATP release upon subsequent mechanical stimulation, and serving as a mechanism of cellular mechano-adaptive memory.

Membrane injury. We have demonstrated that non-lethal cell membrane injury is routine in mechanically stimulated cells. In *vitro*, single cell membrane deformation or fluid shear stress transiently compromised cell membrane integrity in 2-30% of cells, which was repaired within 10-100 s. Consistent with findings observed using *in vitro* models of mechanical stimulation, *in vivo* cyclic compressive tibial loading engendering physiological and supraphysiological strains resulted in transient osteocyte membrane disruption in 5-10% of cells. Interestingly, supraphysiological mechanical strains improved membrane integrity post-stimulation compared to baseline levels. Previously, non-lethal reversible cell wounding was shown to occur in mechanically active tissues, with membrane-impermeable marker uptake reported in 3-20% of cells in skeletal muscle, 3-6% in skin, 25% in cardiac muscle, 2-30% in lung and aorta (7). Consistent with our findings, membrane disruption was reported in 20-60% of long bone osteocytes after *in vivo* treadmill loading (8). The higher prevalence of disruption reported by Yu et al. in long bones was likely a consequence of cumulative dye uptake over 18 h (8), while our study examined membrane disruption over a 20-50 min period. Our data demonstrated that reparable membrane injuries are common and significantly contribute to ATP release from mechanically stimulated osteoblasts and osteocytes.

ATP release. We observed that osteoblasts release 70 ± 24 amol ATP /cell in response to direct membrane deformation and 21 ± 11 to 422 ± 97 amol ATP/cell in response to tFSS, which is consistent with previously reported estimates ranging from 24 ± 1 to 324 ± 59 amol ATP/cell (5, 16-22). Osteoblasts have been reported to release ATP through mechanisms related to vesicular exocytosis (5, 21) and L-type VSCC (5). In osteocytes, hemichannels (17, 23), P2X7 (23) and T-type VSCC (24) have also been implicated. We visualized ATP-containing quinacrine-positive vesicles and directly demonstrated their release upon mechanical stimulation. Surprisingly, the total vesicular ATP content was ~ 94 ± 5 amol ATP/cell, which, considering that only 7.2 ± 1.2% of vesicles were released upon mechanical stimulation, is much less (i.e., <10 amol ATP/cell) than mechanically-induced ATP release. Moreover, treatments that potentiated vesicular release resulted in a surprising decrease in ATP release, while treatments interfering with vesicular release enhanced ATP release. In search for an alternative route of ATP release, we found that mechanically stimulated ATP release was proportional to the extent of membrane injury.

Membrane resealing. Vesicular exocytosis is believed to promote membrane repair via membrane insertion, thereby reducing membrane tension and allowing exposed hydrophobic residues to reseal (7). We have shown that vesicular release was critical for the repair of mechanically-induced membrane injury and that the Ca²⁺/PLC/PKC pathway regulates vesicular exocytosis in osteoblasts. We interrogated the role of vesicular release in the mechanoresponse using NEM, a modulator of vesicular docking machinery (12). While NEM is commonly used as
an inhibitor of vesicular release (5, 25), it has also been shown to increase vesicular release probability (26) through accumulation of readily-releasable vesicles (12). Using real-time imaging, we demonstrated that NEM increased the basal vesicular density and potentiated mechanically-stimulated vesicular exocytosis in osteoblasts. In the presence of NEM, mechanically-induced membrane injury and ATP release were significantly reduced. Vesicular exocytosis has been implicated as a mechanism of ATP release from osteoblasts and osteocytes (5, 17, 21) based on the assumed inhibition of vesicular exocytosis by NEM or vesicular trafficking by brefeldin A, bafilomycin or monensin. However, our data demonstrated that the role of vesicles cannot be inferred without a direct validation of the effect of NEM on vesicular release. In our study, [Ca$^{2+}$]$_e$ depletion abolished mechanically-stimulated vesicular release, interfered with membrane repair and increased ATP release. Calcium is one of the first mechanically-induced signals that initiates downstream responses (3), including exocytosis-mediated membrane repair (27). It was previously shown that during in vivo loading of mouse bone, the number of osteocytes exhibiting [Ca$^{2+}$]$_i$ elevations increased proportionally to the magnitude of applied strain (28). We found that PLC and PKCµ, known downstream targets of Ca$^{2+}$ signaling (29), regulated basal vesicle abundance and membrane resealing. Our study suggests that the osteoporotic phenotype reported in PKCµ-deficient bones (30) may be associated with defective mechanoresponsiveness. Consistent with mechanisms of facilitated membrane resealing (9, 27), we propose that mechanical stimulation results in membrane disruption, which leads to ATP efflux and Ca$^{2+}$ influx, triggering vesicular exocytosis and membrane repair that then limits ATP release (Fig. 7).

**Mechano-adaptive memory.** Our findings suggest that osteoblast mechano-adaptive status may be influenced by PKCµ-vesicular signaling. Activation of PKC prior to mechanical stimulation or priming the vesicular pool for release with NEM resulted in drastic decreases in membrane injury and ATP release in response to subsequent mechanical stimulation. Prior work has demonstrated that mechanical loading induces the production and release of LAMP-1-positive vesicles from osteocytes (LAMP-1 colocalizes with the quinacrine tracer-dye used in this study (31)), which was critical for mechano-adaptive bone formation (32). This mechano-adaptive effect may underlie cellular accommodation in the bone response to mechanical loading previously reported (33), and explain the reduced responsiveness of osteocytes exposed to higher frequency in vivo mechanical loading, compared to lower frequency loading of the same magnitude (28). Our findings suggest that during repeated mechanical stimulation, the priming of PKCµ-vesicular pathway by earlier stimuli will determine osteoblast resilience and responsiveness to subsequent cycles of mechanical stimulation, representing a mechanism for cellular accommodation.

**Concluding remarks.** In this study we established the regulatory mechanisms involved in controlling the amount of ATP released following reversible cellular injury. We have demonstrated that membrane injury and repair are common under physiological stresses in vitro
and in vivo, and determined the critical role for PKC-regulated vesicular exocytosis in bone cell membrane repair. We suggest that rather than delivering ATP to extracellular space, exocytosis of ATP-containing vesicles limits the much larger efflux of intracellular ATP through damaged membranes. We propose a model of biological adaptation to mechanical forces, which directly links mechanosensation through reversible membrane injury and ATP release to the development of adaptive resilience against the destructive potential of mechanical forces. PKC-mediated vesicular release provides a target for potential therapeutic interventions to modulate mechano-responsiveness and mechano-resilience in humans habitually experiencing altered mechanical environment, such as astronauts or paralysis patients.

Materials and Methods

All procedures were approved by McGill’s University’s Animal Care Committee (protocols #2012-7127 and 2016-7821) and complied with the ethical guidelines of the Canadian Council on Animal Care.

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**Solutions and Reagents**

Solutions. Phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, pH 7.4), sterilized by autoclave. Physiological solution (PS; 130 mM NaCl; 5 mM KCl; 1 mM MgCl$_2$; 1 mM CaCl$_2$; 10 mM glucose; 20 mM HEPES, pH 7.6), sterilized by 0.22 µm filtration. In [Ca$^{2+}$]-free PS, CaCl$_2$ was excluded and 10 mM EGTA was added to chelate any residual calcium, sterilized by 0.22 µm filtration. Bioluminescence reaction buffer (0.1 M DTT, 25 mM tricine, 5 mM MgSO$_4$, 0.1 mM EDTA, 0.1 mM Na$_3$PO$_4$, pH 7.8), sterilized by 0.22 µm filtration. RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mg/mL aprotinin, 2 mg/mL leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 0.5 mM Na$_3$VO$_4$), TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Tween 20).

**Pharmacological interventions**

The pharmacological interventions (final concentration, name (abbreviation): molecular target) used in this study were: 10 µM Gadolinium (Gd$^{3+}$): maxi-anion channel, 10 µM Carbenoxolone (Cbx): connexins/pannexins, 10 µM Flufenamic Acid (FFA): connexins, 1 mM 1-Octanol (Oct): connexins, 100 nM GSK 2193874 (GSK): TRPV4, 100 nM HC 067047 (HC): TRPV4, 10 µM Nifedipine (Nif): L-type VSCC, 10 µM ML218 (ML): T-type VSCC, 100 µM Suramin (Sur): P2 receptors (P2R), 100 µM PPADS: P2 receptors, 1 µM A740003 (A7): P2X7 receptor, 10 µM U73122 (U73): PLC, 100 nM Phorbol 12-myristate 13-acetate (PMA): PKC, 1 µM Bisindolylmaleimide II (Bis): PKC, 1 mM N-Ethylmaleimide (NEM): NSF, 1 µM GsMTx-4 (GsM): Piezo1. Unless stated otherwise, cells were treated with drugs for 10 mins prior to experiments. We validated the effects of hemichannel blockers (Fig 4-fig S1) and evaluated the effects of the inhibitors on ATP- or citrate-induced [Ca$^{2+}$]$_i$ responses in Fura2-loaded C2-OB,
cells and on luciferin/luciferase bioluminescence (Fig. 6-fig. S5).

**Cell cultures**

Three osteoblast models were used in this study. All key experiments required to support our proposed model were performed using primary CB-OB cells, however, additional data from another primary source (bone marrow-derived; BM-OB) and cell-line (C2-OB) were reported to emphasize the validity of our findings and demonstrate how different osteoblast models perform in this study.

*Bone marrow-derived osteoblasts (BM-OB):* To obtain bone marrow-derived osteoblasts, 4-6 week C57Bl/6J mice (Charles River) were sacrificed and femur and tibia were collected. Bone pieces were sterilized in 70% EtOH and washed (3x, PBS). Bones were cut in half and placed into longitudinally-cut 1 mL pipette tips, with the exposed marrow end of the bone facing down. Pipette tips were centrifuged in Eppendorf tubes at 12 000 rpm (3x, 30 s) to isolate bone marrow cells. Bone marrow cells were plated at a density of $5 \times 10^4$ cells per cm$^2$ in osteoblastic differentiation medium (αMEM supplemented with 10% FBS, *BMP-2 transfected C2C12 cells (C2-OB):*)

*Compact bone-derived osteoblasts (CB-OB):* Bone-marrow depleted bones, obtained after bone marrow cell isolation, were used as source of CB-OB. Epiphyseal ends of the bone were cut off and the remaining diaphysis were cut into small fragments (<1 mm$^2$ pieces. Bone fragments underwent a three-step enzymatic digest: (i) 10 mL αMEM (serum-free) + 125 µL 0.25% trypsin + 5 µL collagenase P (37°C, 15 min while rotating). (ii) Dispose supernatant and add 10 mL αMEM (serum-free) + 125 µL 0.25% trypsin + 10 µL 100 mg/mL collagenase P (37°C, 30 min while rotating). (iii) Dispose supernatant and add 10 mL αMEM (serum-free) + 125 µL 0.25% trypsin + 100 µL 100mg/mL collagenase II (37°C, 1 hr while rotating, shake vigorously every 10 min). Supernatant from final digest was disposed, bone fragments were rinsed with PBS and plated in 6-well plates in αMEM (10% FBS, 1% sodium pyruvate, 1% penicillin streptomycin and 50 µg/mL ampicillin) for 3-5 days to allow cell outgrowth from fragments. Cells were detached with trypsin, filtered through 40 µm membrane and plated at a density of $1 \times 10^4$ per cm$^2$ in osteoblastic differentiation medium (αMEM supplemented with 10% FBS, 1% sodium pyruvate, 1% penicillin streptomycin and 50 µg/mL ascorbic acid) 2-3 days prior to experiments. Primary osteoblast cultures were sub-cultured for up to 8 passages (1:10 passages).

*BMP-2 transfected C2C12 cells (C2-OB):* The C2C12 cell line (ATCC CRL-1772) stably transfected with BMP-2 (courtesy of Dr. M. Murshed, McGill University) was maintained in DMEM (supplemented with 1% penicillin streptomycin, 1% sodium pyruvate and 10% FBS) at 5% CO$_2$, 37°C humidified atmosphere. Absence of mycoplasma contamination was verified in cryo-preserved stocks of C2-OB cells using the Venor GeM Mycoplasma PCR-based detection
kit (Sigma MP0025). To further authenticate the osteoblast phenotype in the C2-OB cell line, osteogenic differentiation was induced with ascorbic acid (50 µg/mL) and changes in osteogenic gene expression after 7 days of culture were assessed by qRT-PCR. Significant increases in expression of osteoblast marker genes Runx2 (+37%, p=0.02), Alpl (+57%, p=0.03) and Col1a1 (+172%, p<0.001) were observed, while Bmp2 expression was stable overtime, thereby confirming the osteoblastic phenotype in this cell-line. Cells between passages 6 – 10 were used in all experiments. 2-3 days prior to experiments, cells were plated at a density of 3×10^4 cells per cm^2.

**Osteoblast phenotype**

Cells were fixed with formalin (pH 7.4, 8 min) rinsed with PBS (3x). Alkaline phosphatase (ALP) staining solution was prepared by combining 4.5 mg fast red violet salt dissolved in 3.75 mL H_2O and 3.75 mL 0.2 M Tris-HCl, pH 8.3 with 0.75 mg Naphthol AS-MX phosphate disodium salt dissolved in 30 µL N,N-dimethylformamide. Sufficient staining solution was added to each well to ensure entire surface was covered (approx. 500 µL in 35 mm dish) and incubated at room temperature for 15 min, or until red precipitate formed. Cells were washed with H_2O and observed under bright field microscopy. Alkaline phosphate-positive cells were stained pinkish-red, while yellow hue was observed in alkaline phosphatase-negative cells.

**Mechanical stimulation**

*Local membrane deformation of a single cell:* Single osteoblastic cells were stimulated by local membrane deformation with a glass micropipette using a FemtoJet microinjector NI2 (Eppendorf Inc.). The micropipette was positioned approximately 10 µm from the cell membrane at a 45° angle from the horizontal plane and mechanically stimulated at a speed of 250 µm/s with a contact duration of 60 ms. Glass micropipettes were custom-made from glass capillary tubes (1.5 mm outer diameter, King Precision Glass Inc.) using a Sutter Pipette Puller (Flaming/Brown, model P-87).

*Bulk stimulation using tFSS:* 50% of the culture medium volume was manually withdrawn and replaced at an approximate speed of 2 resuspensions per second for an indicated number of x times (for example, 10x tFSS corresponded to 50% volume displacement performed 10 times).

**Intracellular calcium recording and analysis**

Cells plated on glass-bottom plates (MatTek Corporation) were loaded with a ratiometric fluorescent calcium dye Fura2-AM (r.t., 30 min), washed with PS and allowed to acclimatize for 10 min to reduce the effects of mechanical agitation. [Ca^{2+}]_i was recorded at a sampling rate of 2 images per second (2 Hz) using a Nikon T2000 fluorescent inverted microscope with 40x objective (34). Recordings consisted of ~10 s baseline [Ca^{2+}]_i and additional 110-170 s following application of mechanical stimulation or treatment. The excitation wavelength was alternated between 340 and 380 nm using an ultra-high-speed wavelength switching illumination system (Lambda DG-4, Quorum Technologies). Regions of interest (ROI) were manually
defined and the ratio of the fluorescent emission at 510 nm, following 340 and 380 nm excitation (f340/f380), was calculated and exported using the imaging software (Volocity, Improvision). All data were imported into an excel spreadsheet for characterization using a MATLAB algorithm previously described (35). The following parameters were obtained for statistical analysis: amplitude (amp; magnitude of response), activation time (t_{10%-90%}; time between 10% and 90% of maximum response), and decay constant (\tau_{\text{decay}}) of exponential decay region of deactivation phase of transient response. Micropipette stimulation experiments, secondary responsiveness was calculated as the percentage of neighboring cells exhibiting [Ca^{2+}]_i elevations.

**Vesicle Labeling, Imaging, and Analysis**

**Localization:** To visualize the intracellular distribution and colocalization of quinacrine and MANT-ATP, cells were loaded with MANT-ATP (5 \mu M, overnight, 37°C) followed by quinacrine (10 \mu M, 15 min, room temperature). Cells were washed with PS and imaged using a Zeiss LSM 780 laser scanning confocal microscope. An argon and diode laser were used as light sources (ex. 405 nm and 458 nm) and the detection wavelength ranges were set to 410-470 nm and 490-570 nm to ensure separation of overlapping emission spectra, for MANT-ATP and quinacrine, respectively. Images were acquired using a Zeiss Plan-Apochromat 63x/1.40 Oil immersion objective lens. Subsequent analysis was carried out using ImageJ (National Institutes of Health, USA). Vesicular density was determined using ImageJ by counting number of intracellular quinacrine-positive puncta and normalizing count to cell surface area.

**Kinetics:** To study vesicular release kinetics, time-lapse recordings of quinacrine-loaded cells were acquired with a Nikon T2000 fluorescent inverted microscope after a 10 min period of post-stain acclimatization to minimize the effects of mechanical agitation. Quinacrine-rich vesicles were imaged using a 40x objective at a sampling frequency of 2 Hz for 2 min. To minimize the phototoxic effects of quinacrine, the excitation intensity was set to 33% of peak intensity and the shutter was shut in between 100 ms exposures. Image sequences were exported using the imaging software (Velocity, Improvision) for subsequent analysis in ImageJ. To quantify the kinetics of vesicular release, sudden losses of fluorescence were monitored. Vesicles that gradually came in and out of focus did not qualify as release events. SparkSpotter (Babraham Bioinformatics, UK), an ImageJ plugin, was used to identify release events in temporally reversed and contrast-enhanced image stacks, so that the loss of fluorescence would instead be detected as a sudden increase by the spark identifying algorithm. Acquired data was visually evaluated and represented as either release events per second or cumulative vesicular release for indicated time frame, normalized to unit area.

**ATP Measurements**

**Fluorescence recordings of [ATP]_e:** 10 nM D-luciferin- and 2 \mu g/mL fire-fly luciferase-containing physiological solution (PS) was added to C2-OB and extracellular luciferin fluorescence was recorded (380/510 ex/em). Using an ATP standard calibration curve (Fig. 2-
fig. S1), pericellular (<15 µm from stimulated cell) ATP concentrations [ATP] were obtained. The amount of ATP released by individual cells was estimated by fitting the diffusion profile obtained from the fluorescence measurements with the diffusion equation for release from an instantaneous plane source (36).

\[
[\text{ATP}] (x, t) = \frac{M}{2(\pi Dt)^{\frac{1}{2}}} \exp\left(\frac{-x^2}{4Dt}\right)
\]

Where \([\text{ATP}] (x, t)\) is ATP concentration at distance \(x\) from the source, the time \(t\) from the moment of release. \(M\) is the amount of ATP released at \(x = 0, t = 0\). \(D\) is ATP diffusion coefficient, assumed at \(3.3 \times 10^{-6} \text{ cm}^2/\text{s}\) at 22°C (37).

Bioluminescent recordings of [ATP]: Bioluminescence assay was used to measure ATP content in supernatant samples. Luciferin-luciferase reaction solution contained 0.45 µM D-luciferin and 40 µg/mL firefly-luciferase in bioluminescence reaction buffer. 10 µL supernatants were sampled after tFSS stimulation, added into a glass cuvette containing 100 µL of luciferin-luciferase reaction solution, and measured using FB 12 single tube luminometer. Total cellular ATP content was measured in RIPA-lysed samples. [ATP] was estimated using calibration curves (Fig. 6-fig. S5).

Membrane Integrity Assays

Dye leakage: Fura2-loaded osteoblastic cells were mechanically stimulated with a glass micropipette and 510 nm emission intensity was monitored after 340 nm excitation (9). Unless stated otherwise, Fura2 leakage refers to the percentage of cells that were severely injured (sIn; complete loss of intracellular Fura2 fluorescence) following micropipette stimulation.

Dye uptake: 0.08% TB or 10 µM R-dextran were added before or 5 min after mechanical stimulation, washed with PS, and the proportion of dye-positive cells were assessed by bright field microscopy.

Cytosolic leakage: Extracellular lactate dehydrogenase (LDH) was measured using Cytotoxicity Detection Kit\(^\text{PLUS}\). Total cellular LDH content was measured in RIPA-lysed samples.

Cell viability: 1 h after tFSS, AlamarBlue assay was performed according to manufacturer’s protocol. Cell viability was reported as percentage of non-stimulated cells.

In-Vivo Bone Loading

10-week-old female C57Bl/6J mice were injected intraperitoneally with LFTR-Dex either 30 min before or 20 min after loading. In vivo cyclic compressive loading at 600 µε or 1200 µε was applied for 5 min (15). Mice were randomized into two groups, anesthetized, and in vivo cyclic compressive loading was applied to the left tibia (216 cycles at 4Hz the mean mouse locomotor stride frequency (38)), delivering a maximum force of -5.5 N and -11 N, which engenders 600 µε or 1200 µε, respectively at the periosteal surface of the tibia mid-diaphysis in these mice determined by prior in vivo strain gauging studies (39). Previous reports have shown strain levels
of 200-600 µε are engendered on the medial tibia during walking in the mouse (40, 41). Strain levels of 1200 µε are considered supraphysiological resulting in a robust bone formation response after 5 days of mouse tibial loading (15, 42) and altered gene expression after only a single bout of loading (43, 44). Mice ambulated freely after controlled loading, and were euthanized 50 min post dye-injection, weighed (Fig. 5-fig. S1A) and tibiae and calvariae were dissected and fixed in formalin. Tibia lengths were measured (Fig. 5-fig. S1B) and mid-shaft tibiae were cut into fragments, which were immunofluorescently labeled for sclerostin, counterstained with DAPI, and visualized with Nikon T2000 fluorescence inverted microscope. Right non-loaded tibiae were used as an internal control. Female mice were used instead of males to minimize in-cage aggression and related variations in mechanical-loading. Experimenter was blinded during image analysis.

**Immunoblotting**

Cell lysates were extracted in RIPA lysis buffer and centrifuged (12 000 rpm, 10 min, 4°C). Supernatants were collected and protein concentration were determined using Quant-iT protein assay kit (Invitrogen). 70 µg cell lysates were separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane (0.45 µm, 162-0115, Bio-Rat) using a 10 mM sodium borate buffer. The membranes were blocked in 5% BSA in TBST buffer (1 h., room temperature) followed by overnight incubation at 4°C with primary antibodies (1:1000 dilution, 5% BSA in TBST) for phosphorylated PKC isoforms: p-PKC (pan; βII Ser660), p-PKCδ/θ (Ser643/676), p-PKD/PKCµ (Ser744/748) and p-PKCζ/λ (Thr410/403), or for total PKC isoforms: PKCα, PKCδ, PKD/PKCµ and PKCζ. Blots were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature (1:1000 dilution, 5% BSA in TBST) and visualized with a chemiluminescence system.

**GAP Junction functional assay**

Cells were scraped in the presence of GAP-junction permeable dye Lucifer yellow (LY, 10 µM), incubated for 2 min (37°C), rinsed and fixed with formalin (pH 7.4, 8 min). Dye transfer was visualized using wide-field epifluorescence microscopy and cell-cell coupling was visually determined by bright field microscopy. Cells that were not initially scraped but were physically coupled and LY-positive after two minutes of staining were reported as a percentage of total cells that were physically coupled to scraped neighbors.

**Statistical Analysis**

Data are representative results or means ± standard errors (S.E.M). Curve fittings and [Ca\(^{2+}\)]\(_i\) transient characterization was done in MATLAB (MathWorks). Sample sizes for in vitro experiments indicate the total number of biological replicates (specified in figure legends as number of stimulated cells or number of independent cultures, which were isolated from different mice for primary OB, or represent different plating dates for C2-OB) pooled across a minimum of 3 independent experiments. For in vivo experiments, sample sizes indicate number of animals. Statistical significance was assessed by ANOVA followed by Bonferroni post-hoc
test, significance levels are reported as single symbol: *p<0.05; double symbol **p<0.01 or triple symbol ***p<0.001.

References


42. Brodt, M.D. and M.J. Silva, Aged mice have enhanced endocortical response and normal periosteal response compared with young-adult mice following 1 week of axial tibial compression. *J Bone Miner Res.* 25(9), 2006-2015 (2010). [https://doi.org/10.1002/jbmr.96](https://doi.org/10.1002/jbmr.96)


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Figures

Figure 1. Osteoblasts are mechanosensitive (A-C) Single Fura2-loaded C2-OB (A), CB-OB (B) or BM-OB (C) (Top: ALP staning) were stimulated with micropipette and [Ca$$^{2+}$$], was recorded (Fig. 1-video 1). Bottom: representative traces (black), means ± SEM (red) (D) Amplitudes of mechanically-evoked [Ca$$^{2+}$$], transients in osteoblasts pretreated with vehicle, Nif, Sur or PPADS (see materials & methods for abbreviations, targets and doses). Means ± SEM, n = 6-15 stimulated cells, normalized to vehicle. (E, F) Contribution of L-type VSCC (E, n = 10) and P2 receptors (F, n = 12) to mechanically-evoked [Ca$$^{2+}$$], transients in C2-OB as comparison and difference (Insets) of mean ± SEM of vehicle and treatment [Ca$$^{2+}$$], transients. (G) [Ca$$^{2+}$$], in the stimulated (primary) and neighboring (secondary) C2-OB. Top, pseudocolor of 340/380 ratio, white arrows: directions of signal propagation; Bottom, [Ca$$^{2+}$$], transients, red arrows: Top panel time points. (H) Secondary responsiveness in cultures pretreated with vehicle, Sur, PPADS, Cbx, FFA or Oct. Means ± SEM, n = 7-21 stimulated primary responses, normalized to vehicle. (H) [Ca$$^{2+}$$], transients induced by mechanical stimulation of single C2-OB (left, red curve) or puff application of 10 µM ATP (right). For figure 1, *significance between treatment and control by ANOVA. Source data for Fig. 1 is provided in Fig. 1-source data 1.

Figure 2. ATP release following mechanical stimulation of a single osteoblast. (A) Fura2-loaded C2-OB bathed in luciferin/luciferase containing PS was micropipette-stimulated. Top, 380 ex/510 em images before (a) and after (b) stimulation of the cell (red outline); Bottom, intracellular (IC) Fura2 fluorescence [I$$^i_{\text{fura2}}$$] of primary (red) and secondary (orange) responders and extracellular (EC) luciferin fluorescence [I$$^e_{\text{luciferin}}$$] (green). (B) Box plot of [ATP]$$^e$$ in pericellular region of stimulated C2-OB, black markers: independent observations, n = 13 stimulated cells. (C) Correlation between [ATP]$$^e$$ and percentage of secondary responders observed in C2-OB (n = 20 stimulated primary responses), dashed line: linear regression. Source data for Fig. 2 is provided in Fig. 2-source data 1.

Figure 3. Vesicular ATP released upon mechanical stimulation is not the primary source of extracellular ATP. (A) Confocal images of CB-OB loaded with quinacrine and MANT-ATP, Fig 3-videos 1-3. (B) Changes in quinacrine-loaded CB-OB treated with ionomycin (left) or stimulated with micropipette (right, region of interest in white). (C) Vesicular release event in CB-OB (top) coincides with a sudden drop in granular quinacrine fluorescence (bottom), Fig 3-videos 4-6. (D) Kinetics of vesicular release from CB-OB stimulated by micropipette at 0 s, n = 37 stimulated cells. (E-H) Quinacrine- (E, F) or Fura2- (G, H) loaded CB-OB were pre-treated (10 min) with vehicle, ionomycin (iono) or NEM or placed in [Ca$$^{2+}$$]-depleted physiological solution, and micropipette-stimulated when indicated (+). Vesicular density (E) and cumulative release (F), secondary responsiveness (G) and [Ca$$^{2+}$$] response activation rates (H) were determined, n = 7-37 primary cells. (I) tFSS was applied by replacing 50% media volume n
times. (J, K) ATP released per cell (left axis) or as percent of cellular ATP content (right axis) was measured following CB-OB stimulation by tFSS (J, black dashed line: rational function fit; red dashed line: corresponding asymptote) or after indicated pre-treatments followed by tFSS (10x media displacements, +) (K), n = 6-8 independent cultures. For figure 3, means ± SEM, *significance compared to vehicle (E-H), basal ATP release (J) or to tFSS-stimulated vehicle (K) by ANOVA. Source data for Fig. 3 is provided in Fig. 3-source data 1.

Figure 4. Mechanical stimuli regularly and reversibly compromise the integrity of osteoblast cell membrane. (A-F) Membrane permeability in micropipette-stimulated Fura2-loaded osteoblasts. (A) Top: Schematic of dye-leakage assay. Bottom: 340 ex/510 em before (a) and after (b) CB-OB stimulation (white outline). (B) Representative Fura2 traces in C2-OB with minor (mIn), intermediate (iIn) or severe (sIn) cell injury and relative occurrence frequency (Right, n = 35-40 stimulated cells). (C) C2-OB [Ca2+]i responses and differences (Inset) for mIn (green), iIn (orange) and sIn (red), n = 8-14 stimulated cells. (D-F) Primary [Ca2+]i response amplitudes (D) and decay constants (E), and secondary responsiveness (F) in CB-OB (black; n = 40 stimulated cells) or C2-OB (white; n = 35 stimulated cells), grouped by cell injury status. (G-K) Membrane permeability in tFSS-stimulated osteoblasts. (G) Top: schematic of dye-uptake assay. Bottom: CB-OB stained with TB (top) or R-dextran (bottom) prior to (0 s, left) or after (300 s, right) tFSS application (10x). Uptake of TB (H) or R-dextran (I) added prior to (0 s) or after (300 s) tFSS stimulation of CB-OB (n = 4 independent cultures). (J) Leakage of LDH from CB-OB 5 min after tFSS (n = 8 independent cultures, normalized to total LDH). (K) Viability of C2-OB 1 h after tFSS assessed by alamarBlue (n = 8-16 independent cultures). (L) Uptake of TB added at indicated times after micropipette-stimulation of C2-OB (a, n = 4-23 stimulated cells) compared to relative frequencies of mIn, iIn and sIn assessed by Fura2-leakage assay (b). (M) Calculated minimum membrane lesion radius Rmin required for permeability to LDH, R-dextran, TB, Fura2 and ATP. For figure 4, means ± SEM, dashed lines – linear regression, *significance by ANOVA. Source data for Fig. 4 is provided in Fig. 4-source data 1.

Figure 5. Osteocyte membranes are reversibly disrupted during in vivo cyclic compressive loading of the tibia. (A, B) Left tibia of anesthetized mouse was positioned in loading device as shown on the picture (A) and schematic (B). Arrows indicate direction of load. (C) The triangle waveform included 0.15 s symmetric active loading/unloading, with a 0.1 s rest phase (-1 N) between load cycles and a 5 s rest inserted between every four cycles. A maximum force of -5.5 N or -11 N was applied, which engenders 600 µε or 1200 µε, respectively at the periosteal surface of the tibia mid-diaphysis in these mice. (D) Experimental design schematic: animals were injected with LFTR-Dex 30 min before (red) or 20 min after (blue) in vivo cyclic tibial loading (5 min). (E) Proportion of cells exhibiting LFTR-Dex uptake in calvariae (n = 5 animals) and control tibiae (n = 20 from 10 animals), normalized to average total cell number, means ± SEM, **p<0.01. (F) Dye uptake in tibia injected before (red) or after (blue) loading at 600 µε or 1200 µε, means ± SEM, n = 5/strain/dye protocol, normalized to non-loaded contralateral tibia,
*significance compared to non-loaded tibia, ††significance between dye protocols, by ANOVA.

(G) Contrast enhanced images of dye uptake in control (left), and 1200 µε loaded tibia injected with dye before (middle) or after (right) loading. Source data for Fig. 5 is provided in Fig. 5-source data 1.

**Figure 6. Membrane resealing depends on PKC-regulated vesicular exocytosis.** (A-D) CB-OB were pretreated with vehicle, PMA or Bis, and membrane injury (A, B), vesicular exocytosis (C) and ATP release (D) were assessed. (A) TB uptake at 0 and 300 s after tFSS (10x), n = 5-8 independent cultures. (B) Fura2 leakage, determined as percentage of sIn cells after micropipette-stimulation, n=9-16 stimulated cells. (C) Cumulative vesicular release over 100 s after micropipette-stimulation, n=6-10 stimulated cells. (D) ATP release over 60 s following 10x tFSS stimulation, n=8 independent cultures. (E) Immunoblotting for PKC isoform phosphorylation in CB-OB treated with vehicle, PMA or Bis (30 min); or 30 s, 90 s or 300 s after tFSS (10x). Shown are immunoblots (left, complete gels in Fig S8) and densitometry (right) of phosphorylated and total conventional (pPKC pan), atypical (pPKC ζ/λ), novel (pPKC δ/θ), and PKD/PKCµ isoforms. Phosphorylated PKC isoforms were normalized by total PKC levels and reported relative to vehicle, n=3 independent cultures. (F-H) Relationships between vesicular release (pooled data from vesicular release experiments), membrane disruption (pooled from membrane integrity experiments) and ATP release (pooled from 10x tFSS experiments) following mechanical stimulation of CB-OB, solid line - regression. For figure 6, data are means ± SEM, *significance compared to vehicle (0 s vehicle for A), † significance compared to 300 s vehicle (A) and #significance of indicated comparisons (A), by ANOVA or by regression. Source data for Fig. 6 is provided in Fig. 6-source data 1.

**Figure 7. Proposed model for mechanically-stimulated ATP release in osteoblasts.** Resting Osteoblasts: Under basal conditions, the cell membrane is intact and intracellular free calcium levels and PLC/PKC signalling are minimal. Mechanical injury: Mechanical stimulation results in disruption of the cellular membrane, which leads to the influx of calcium, activation of PLC/PKC signalling, and the efflux of ATP. Membrane repair: The disrupted membrane is rapidly repaired through a process involving Ca²⁺/PLC/PKC-dependent vesicular exocytosis, thereby limiting ATP release. Cellular adaptation: Elevated PKC levels result in priming of the vesicular pathway, likely regulating cellular resilience and responsiveness to subsequent cycles of mechanical stimulation.

**Figure Supplements**

Fig. 2-fig. S1. Real-time ATP measurement assay using fluorescent properties of luciferin-luciferase. (A) 1 µM ATP was added to luciferin in presence or absence of luciferase and fluorescence was recorded at 380 ex/510 em; normalized to basal luciferin fluorescence. (B)
[ATP]_e dose dependence of luciferin-luciferase fluorescence change from baseline. Means ± SEM, n = 3 independent recordings, *dashed line: linear regression, ***p<0.001.

**Fig. 3-fig. S1. Involvement of conductive channels in osteoblast response to mechanical stimulation.** (A) Amplitudes of mechanically-evoked [Ca^{2+}]_i transients in osteoblasts pretreated with vehicle, Gd^{3+}, FFA, GSK, HC, Nif, ML, Sur and PPADS. Means ± SEM, n= 5-15 stimulated cells, normalized to vehicle. (B) CB-OB cells were stimulated by tFSS (10x) following pre-treatment with conductive channel inhibitors Gd^{3+}, GSK, HC, Nif, ML, A7, PPADs and GsM. Means ± SEM attomoles ATP released per cell over 60 s after stimulation, n = 6-8 separate cultures, compared to vehicle. *p<0.05, **p<0.01 and ***p<0.001 indicate significance of treatment condition compared to vehicle, assessed by ANOVA followed by post-hoc Bonferroni test.

**Fig. 4-fig. S1. Involvement of hemichannels in ATP release and membrane resealing in murine osteoblasts (A-B)** To demonstrate the presence of functional GAP-junctions and validate GAP-junction/hemi channel blockers, C2-OB were pretreated (10 min) with vehicle (n = 22), Cbx (n = 11), FFA (n = 6) and Oct (n = 10) and scraped and stained with LY. Sample sizes are regions of interest imaged from 3 independent cultures. Fluorescence (A, *top*) and bright field (A, *bottom*) images show dye transfer and cell-cell coupling, respectively. Mean ± SEM percentage of cells that were not initially scraped, but were coupled and LY-positive following LY staining, normalized to vehicle (B). (C-D) Fura2-loaded CB-OB pretreated with GAP-junction/hemi-channel blockers were mechanically stimulated with glass micropipette and [Ca^{2+}]_i elevation amplitude (C, mean ± SEM amplitude, normalized to vehicle) and percentage of sIn cells (D, mean ± SEM percentage) was determined (n=6-9 stimulated cells). (E-F) Membrane injury of CB-OB following tFSS (10x resuspensions) was assessed by T.B. uptake at 0 and 300s (E; n = 5-8 separate cultures, means ± SEM), and ATP release ([ATP]_e) was measured using bioluminescence assay (F; n = 6 separate cultures, means ± SEM attomoles released per cell over 60 s after simulation) following pre-treatment with GAP-junction/hemichannel blockers. Comparisons to vehicle; *p<0.05, **p<0.01 and ***p<0.001, or as specified; #p<0.05, ##p<0.01 and ###p<0.001, indicate significance assessed by ANOVA followed by post-hoc Bonferroni test.

**Fig. 5-fig. S1. in vivo cyclic compressive loading was applied to the left tibia.** (A) Average weights of 10-week-old female C57Bl/6J mice for each experimental cohort. Mean ± SD, n=5 animals per group. (B) Impact of loading on tibial length compared to contralateral non-loaded tibia for 600 µε (n = 10 animals) and 1200 µε (n = 10 animals) loading regime. For (A) and (B) no significant difference was demonstrated by ANOVA. (C) Immunofluorescence images of nuclear DAPI (left), anti-sclerostin (middle) and LFTR-Dex uptake (right) in non-loaded tibia. Magnified regions are contrast-enhanced.

**Fig. 6-fig. S1. PKC regulates membrane resealing and ATP release in C2-OB cell line.** (A-C) C2-OB were pre-treated (30 min) with vehicle, PMA or Bis. (A) Membrane permeability of micropipette-stimulated Fura2-loaded C2-OB was assessed by dye-leakage. Means ± SEM
percentage of sIn cells, n = 11 stimulated cells. (B) Membrane permeability of C2-OB stimulated with tFSS (10x resuspensions) was assessed by staining at 0 or 300s with T.B. Means ± SEM percentage of dye-positive cells, n = 7 separate cultures. (C) Amount of ATP released ([ATP]i) from tFSS-stimulated (10x resuspensions) C2-OB was assessed by bioluminescence assay. Means ± SEM attomoles ATP release per cell, 60 s after stimulation. Compared to vehicle (unless otherwise specified), **p<0.01 and ***p<0.001 indicate significance assessed by ANOVA followed by post-hoc Bonferroni test.

**Fig. 6-fig. S2. Membrane resealing, vesicular exocytosis and ATP release are Ca2+/PLC-dependent processes.** (A-E) CB-OB were pretreated with vehicle, calcium-depleted physiological solution (-Ca2+), U73, NEM, PMA or Bis where indicated, and mechanically-induced membrane injury (A, B), amplitude of [Ca2+]i elevations (C), vesicular exocytosis (D) and ATP release (E) were assessed. (A) TB uptake at 0 and 300 s after tFSS (10x), n = 5-8 separate cultures. (B) Fura2 leakage following micropipette stimulation, determined as percentage of sIn cells after micropipette-stimulation, n = 9-16 stimulated cells. (C) Amplitudes of micropipette-stimulated [Ca2+]i elevations in Fura2-loaded C2-OB and CB-OB cells, n = 9-16 stimulated cells. (D) Cumulative vesicular release over 100 s after micropipette-stimulation, n = 6-10 stimulated cells. (E) ATP release over 60 s following 10x tFSS stimulation, n = 8 separate cultures. For Fig S6, data are means ± SEM, ***significance compared to vehicle (0 s vehicle for A), ††significance compared to 300 s vehicle (A) and ** significance of indicated comparisons (A), by ANOVA or by regression.

**Fig. 6-fig. S3. Basal vesicular density and mechanically-stimulated vesicular release in compact bone-derived osteoblasts.** (A-B) Quinacrine-loaded CB-OB were pretreated (30 min) with PMA, Bis, PMA + Bis, U73, U73 + PMA, [Ca2+]c-free PS, [Ca2+]c-free PS + PMA and NEM. Basal vesicular density (A) and cumulative vesicular release in response to stimulation by glass micropipette (B) were determined. Mean ± SEM vesicular density or cumulative vesicular release, normalized to unit area (n = 11-16 stimulated cells). Comparisons with vehicle; *p<0.05, **p<0.01 and ***p<0.001, or as specified; *#p<0.05, **#p<0.01 and ###p<0.001, indicate significance assessed by ANOVA followed by post-hoc Bonferroni test.

**Fig. 6-fig. S4. Immunoblot analysis of PKC isoforms.** (A-D) Immunoblots for phospho- and total- conventional PKC (A), novel PKC (B), PKC/PKCµ (C) and atypical PKC (D) isoforms, and GAPDH and β-tubulin in cell lysates extracted from CB-OB treated with vehicle, PMA or Bis (30 min); or 30 s, 90 s or 300 s after tFSS (10x). (E-G) GAPDH (E) and β-tubulin (F) band intensities were averaged and used to normalize total PKC levels (G). Differences in loading controls and total PKC levels were assessed by ANOVA and p-values are reported, n = 3 cultures isolated from different mice.

**Fig. 6-fig. S5. Effect of drug treatments on [Ca2+]i elevations and ATP bioluminescence assay.** (A, B) Effect of drugs on P2 receptor-mediated [Ca2+]i elevation amplitude (A) and responsiveness (B). 1 µM ATP was applied to Fura2-loaded C2-OB pre-treated for 10 mins with vehicle (n = 30), GSK (n = 28), HC (n = 30), Gd3+ (n = 18), Nif (n = 46), ML (n = 45), Sur (n =
51), PPADS (n = 36), Cbx (n = 47), FFA (n = 45), Oct (n = 39) or 30 mins with [Ca\(^{2+}\)]\(_i\)-free PS (n = 62), U73 (n = 29), PMA (n = 18), or Bis (n = 48). Mean ± SEM response normalized to vehicle. Compared to vehicle, *p<0.05, **p<0.01 and ***p<0.001 indicate significance assessed by ANOVA followed by post-hoc Bonferroni test. (C) Effect of drugs on citrate-mediated [Ca\(^{2+}\)]\(_i\) elevation amplitude. 1 mM citrate was applied as TRP agonist to Fura2-loaded C2-OB pretreated for 10 mins with GSK (n = 48), HC (n = 18) or Gd\(^{3+}\) (n = 29). Mean ± SEM amplitude normalized to vehicle condition. For A-C, sample sizes are number of quantified cells from at least 3 independent cultures. (D) Calibration curve relating ATP standard concentrations to measured bioluminescence. Mean ± SEM relative light units per sec (RLU/s). Solid line, linear regression. (E) Effect of drugs on ATP bioluminescence assay. 10 nM ATP-dependent luciferin/luciferase bioluminescence was measured in the presence of PMA, Bis, [Ca\(^{2+}\)]\(_i\)-free PS, U73, Cbx, FFA, Oct, NEM, Gd\(^{3+}\), PPADS, Nif, ML, A7, GSK, HC, or GsM. Mean ± SEM RLU/s as percentage of vehicle condition. Compared to vehicle, **p<0.01 and ***p<0.001 indicate significance assessed by ANOVA followed by post-hoc Bonferroni test.

**Video Supplements**

**Fig. 1-video 1.** Single Fura2-loaded C2-OB was stimulated with micropipette and [Ca\(^{2+}\)]\(_i\) was recorded. Pseudocolor corresponds to 340/380 ratio.

**Fig. 3-video 1.** Confocal Z-stack of quinacrine- (left) and MANT-ATP (middle)-loaded CB-OB cells with merged images (right), ex. 1.

**Fig. 3-video 2.** Confocal Z-stack of quinacrine- (left) and MANT-ATP (middle)-loaded CB-OB cells with merged images (right), ex. 2.

**Fig. 3-video 3.** Confocal Z-stack of quinacrine- (left) and MANT-ATP (middle)-loaded CB-OB cells with merged images (right), ex. 3.

**Fig. 3-video 4.** Single quinacrine-loaded CB-OB was stimulated with micropipette and vesicular release events were recorded, ex. 1.

**Fig. 3-video 5.** Single quinacrine-loaded CB-OB was stimulated with micropipette and vesicular release events were recorded, ex. 2.

**Fig. 3-video 6.** Single quinacrine-loaded CB-OB was stimulated with micropipette and vesicular release events were recorded, ex. 3.

**Fig. 5-video 1.** Recording of in vivo cyclic compressive loading being applied to left tibia of anesthetized C57Bl/6J mouse.
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▲ ATP □ vesicles ▼ cell membrane ▲ mechanical stimulus