P2Y Purinoceptors Are Responsible for Oscillatory Fluid Flow-induced Intracellular Calcium Mobilization in Osteoblastic Cells*

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We previously found that oscillatory fluid flow activated MC3T3-E1 osteoblastic cell Ca2++ mobilization via the inositol 1,4,5-trisphosphate pathway in the presence of 2% fetal bovine serum (FBS). However, the molecular mechanism of fluid flow-induced Ca2++, mobilization is unknown. In this study, we first demonstrated that oscillatory fluid flow in the absence of FBS failed to increase [Ca2++]i in MC3T3-E1 cells. A pyrase (10 units/ml), which rapidly hydrolyzes 5’ nucleotide triphosphates to monophosphates, prevented the fluid flow induced increases in [Ca2++]i in the presence of FBS. Adding ATP or UTP to flow medium without FBS restored the ability of fluid flow to increase [Ca2++]i, suggesting that ATP or UTP may mediate the effect of fluid flow on [Ca2++]i. Furthermore, adenosine, AMP, UDP, or adenosine 5’-O-(3-thiotriphosphate) did not induce Ca2++ mobilization under oscillatory fluid flow without FBS. Pyridoxal phosphate 6-azophenyl-2,4-disulfonic acid, an antagonist of P2X purinoceptors, did not alter the effect of fluid flow on the Ca2++ response, whereas pertussis toxin, a Gαi protein inhibitor, inhibited fluid flow-induced increases in [Ca2++]i in the presence of 2% FBS. Thus, by the process of elimination, our data suggest that P2Y purinoceptors (P2Y2 or P2Y4) are involved in the Ca2++ response to fluid flow. Finally, a decreased percentage of MC3T3-E1 osteoblastic cells treated with P2Y2 antisense oligodeoxynucleotides responded to fluid flow with an increase in [Ca2++]i, and an increased percentage of ROS 17/2.8 cells, which do not normally express P2Y2 purinoceptors, transfected with P2Y2 purinoceptors responded to fluid flow in the presence of 2% FBS, confirming that P2Y2 purinoceptors are responsible for oscillatory fluid flow-induced Ca2++ mobilization. Our findings shed new light of the molecular mechanisms responsible for oscillatory fluid flow-induced Ca2++ mobilization in osteoblastic cells.

It is well known that mechanical loading of bone results in a variety of biophysical signals that may affect bone cellular metabolism and differentiation (1–3). One of these biophysical signals, extracellular fluid flow, has been demonstrated to be a potent stimulator of osteoblastic cell metabolic activity, differentiation, extracellular matrix protein production, and gene expression (4–8). Recently we reported that oscillatory fluid flow in the presence of 2% fetal bovine serum (FBS),1 activated MC3T3-E1 osteoblastic cell intracellular calcium (Ca2++), mobilization via the inositol 1,4,5-trisphosphate pathway and increased steady state levels of osteopontin, a bone extracellular matrix protein, mRNA in a Ca2++-dependent manner (9). We and others (10) also found that extracellular signaling molecules present in FBS are necessary for fluid flow-induced increases in intracellular calcium ion concentration ([Ca2++]i) in bone cells. However, the nature of these signaling molecules, their receptors, and their roles in oscillatory fluid flow-induced Ca2++ mobilization are still unclear.

Ca2++ is an early response second messenger that plays an important role in a number of intracellular signaling pathways and is typically observed to increase dramatically within seconds of stimulation. As a second messenger, Ca2++ transduces extracellular changes (i.e. first messenger) to the cell interior and potentially to the genome and is important in the regulation of a variety of cellular functions (11). For instance, we previously demonstrated that oscillatory fluid flow regulates steady state osteopontin mRNA levels in a manner dependent on Ca2++, mobilization and via extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways (9). In addition, other studies demonstrated that oscillations in intracellular Ca2++ regulate expression of transcription factors such as NFAT, NFκB, and Oct/OAP, which may be involved in cellular differentiation (12, 13). Thus, understanding the molecular mechanism of fluid-induced Ca2++ mobilization is a very important step elucidating mechanotransduction pathways in osteoblastic cells.

It is well documented that extracellular nucleotides such as ATP and UTP induce a broad spectrum of cell responses including Ca2++ mobilization. These responses include excitation of sympathetic neurons, muscle cell proliferation, endothelial cell adhesion, spermiogenesis, acid-base equilibrium in intestinal epithelial cells, and mucociliary clearance in normal cystic fibrosis airway epithelia (14–16). It is also recognized that extracellular nucleotides act as important signaling molecules for cell-cell communication among bone cells (17). Additionally there is increasing evidence that extracellular nucleotides play

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¶ The abbreviations used are: FBS, fetal bovine serum; ATPγS, adenosine 5’-O-(3-thiotriphosphate); Ca2++, intracellular calcium; MEM, minimal essential medium; Nm2+ Newtons/meter2; P2Y2-AS, antisense oligodeoxynucleotides of P2Y2; P2Y2-SCR, scrambled control oligodeoxynucleotides of P2Y2.

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an important role in bone remodeling (18). Therefore, extracellular nucleotides are potential candidates responsible for fluid flow-induced Ca\(^{2+}\) mobilization in bone cells.

The extracellular nucleotides ATP and UTP are known to exert their effects via a family of specific receptors termed P2 purinergic receptors (purinoceptors) (19, 20). The P2 purinoceptors are divided into the P2X receptors, which are ligand-gated ion channels, and the P2Y receptors, which are G protein-coupled receptors. Up to seven P2X subtypes (P2X1–P2X7) and several types of P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11) have been cloned (19, 20). P2 purinoceptors are expressed in osteoblastic cells of rat and human origin (21), and some studies have demonstrated the extracellular nucleotides such as ATP and ADP increase osteoblastic cell proliferation and differentiation through specific P2 purinoceptors (22, 23).

Thus, in this study we hypothesized that extracellular nucleotides such as ATP and UTP and their specific purinoceptors mediate oscillatory fluid flow-induced Ca\(^{2+}\) mobilization in osteoblastic cells. To test our hypothesis, we first utilized apyrase, an enzyme that quickly hydrolyzes 5’ nucleotide triphosphates to monophosphates, to examine whether extracellular 5’ nucleotide triphosphates such as ATP and UTP are necessary for oscillatory fluid flow to induce Ca\(^{2+}\) mobilization in MC3T3-E1 osteoblastic cells. Secondly, different nucleotides and their agonists or antagonists were added to the fluid medium to investigate the possible involvement of different nucleotides responsible for oscillatory fluid flow-induced Ca\(^{2+}\) mobilization. We identified P2Y as the purinoceptors involved in the fluid flow-induced Ca\(^{2+}\) response. Finally, we employed molecular techniques to manipulate functional purinoceptor expression in osteoblastic cells to confirm that a specific purinoceptor, P2Y2, is involved in oscillatory fluid flow-induced Ca\(^{2+}\) responses.

**MATERIALS AND METHODS**

**Cell Culture**—The mouse osteoblastic cell line MC3T3-E1 was cultured in minimal essential medium supplemented with 10% FBS (Hyclone, Logan, UT) and 1% penicillin and streptomycin (Invitrogen). The rat osteoblastic cell line R0S 17/2.8, which does not normally express P2Y2 receptors (24), was grown in minimal essential medium (MEM) supplemented with 10% heat-inactivated calf serum (Hyclone), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (Invitrogen), 2 mM L-glutamine, and 1% penicillin and streptomycin. ROS 17/2.8 cells transfected with human P2Y2 purinoceptors (ROS/P2Y2) were cultured in the same media as ROS 17/2.8 but with 400 µg/ml Geneticin (Invitrogen) added. All cells were maintained in a humidified incubator at 37°C with 5% CO\(_2\). 0.85 × 10\(^6\) cells were subcultured on quartz slides for 3 min before the application of oscillatory fluid flow. The fluid flow chamber employed in this study is a parallel plate design, and the flow delivery device generated 1 Hz sinusoidally oscillating flow (peak shear stress 2 N/m\(^2\)). Both have been described in previous studies (9, 25).

**Calcium Imaging**—[Ca\(^{2+}\)]\(_i\) was quantified with the fluorescent dye fura-2. Fura-2 exhibits a shift in absorption when bound to Ca\(^{2+}\) such that the emission intensity when illuminated with ultraviolet light increases with [Ca\(^{2+}\)]\(_i\), at a wavelength of 340 nm and decreases with [Ca\(^{2+}\)]\(_i\), at 380 nm. The ratio of light intensity between the two wavelengths corresponds to [Ca\(^{2+}\)]\(_i\). A calibration curve of intensity ratio and [Ca\(^{2+}\)]\(_i\) was obtained using fura-2 in buffered calcium standards supplied by the manufacturer (Molecular Probes, Inc., Eugene, OR). Pre-confluent (80%) cells were washed with MEM-α and 2% FBS at 37°C, incubated with a 10 µM fura-2-acetoxymethyl ester (Molecular Probes) solution for 30 min at 37°C, and washed again with fresh MEM-α and 2% FBS. The quartz slide with the cells was mounted on a parallel plate flow chamber filled with the experimental media. The chamber was placed on an inverted fluorescence microscope (Nikon, Melville, NY) and left undisturbed for 30 min before experiments. Cell assemblies were illuminated at wavelengths of 340 and 380 nm in turn. Emitted light was passed through a 510-nm interference filter and detected with an intensified charge-coupled device camera (International LDT, Sterling, VA). Images were recorded once every 2 s and analyzed using image analysis software (Metamorph; Universal Imaging, West Chester, PA). Basal [Ca\(^{2+}\)]\(_i\) was sampled for 3 min and followed by 3 min of oscillatory fluid flow.

**Oligodeoxynucleotide Treatment**—Antisense oligodeoxynucleotides directed against P2Y2, 5’-CAG GTG TGC TGC CAT CAT-3’ (P2Y2-AS), which had been demonstrated to inhibit P2Y2 purinoceptor function in astrocytes (26), were employed to target the P2Y2 purinoceptor on MC3T3-E1 cells. Scrambled control oligodeoxynucleotides of P2Y2 5’-GTG CTC GTA GTT ACC-3’ (P2Y2-SCR), were designed and synthesized at the Core Facilities of Pennsylvania State University College of Medicine. 8 × 10\(^4\) MC3T3-E1 cells were precultured for 24 h and incubated in the presence of P2Y2-AS or P2Y2-SCR with LipofectAMINE PLUS (Invitrogen) for 2.5 h. After transfection, cells were cultured in fresh culture medium for another 24 h before fluid flow experiments. Cellular uptake of antisense oligodeoxynucleotides, which were labeled with fluorescein isothiocyanate at their 5’-end (Synthetic Genetics Inc., San Diego, CA), was verified by observation of fluorescein isothiocyanate with a fluorescence microscope.

**Pharmacological Agents**—Five nucleotides (ATP, UTP, GTP, CTP, and TTP) were employed as extracellular signaling molecules in flow experiments. Apyrase (100 units/ml), an enzyme that rapidly hydrolyzes 5’ nucleotide triphosphates to monophosphates, was used to inhibit the nucleotides effects (27) and was applied 30 min before fluid flow. Different purinoceptor agonists (ADP, UDP, ATP-S, and adenosine) and antagonist (pyridoxal phosphate 6-azophenyl-2’-4’-disulfonic acid) were added to the experimental media to identify which specific purinoceptors were expressed on the MC3T3-E1 cells. Pertussis toxin (Calbio-chen) was used to assess the role of G\(_i\)-proteins in the Ca\(^{2+}\) response to fluid flow. Cells were exposed to 200 ng/ml pertussis toxin in media for 90 min before fluid flow. To remove triphosphate contamination from diphosphate nucleotides, 1 mM stock solutions of UDP and ADP were pretreated with 10 units/ml hexokinase for 30 min at 37°C in the presence of 5 mM glucose (28). All the reagents were from Sigma unless otherwise indicated.

**Data Analysis**—We used a numerical procedure from mechanical analysis known as Rainflow cycle counting to identify calcium oscillations (29). Briefly, this technique identifies complete cycles or oscillations in the time history data even when they are superimposed upon each other and, therefore, can be used to distinguish and quantify Ca\(^{2+}\) responses from background noise. We defined a response as an oscillation in [Ca\(^{2+}\)]\(_i\), at least 2-fold greater than that of the average base-line level of nontreated cells. Base-line [Ca\(^{2+}\)]\(_i\) data were recorded for each slide for 3 min before the application of oscillatory fluid flow.

Data were expressed as the mean ± S.E. To compare observations from no flow and flow responses, a two-sample Student’s t test was used in which sample variance was not assumed to be equal. To compare observations from more than two groups, a one-way analysis of variance was employed followed by a Bonferroni selected-pairs multiple comparisons test (Instat, GraphPad Software Inc., San Diego, CA). p < 0.05 was considered statistically significant.

**RESULTS**

**Extracellular Nucleotides Are Necessary for Fluid Flow-Induced Ca\(^{2+}\) Mobilization**—To investigate the role of extracellular molecules in FBS or induced by FBS in fluid flow-induced Ca\(^{2+}\) mobilization, MC3T3-E1 cells were exposed to flow media with or without FBS (Fig. 1A). In the absence of 2% FBS, oscillatory fluid flow (2N/m\(^2\), 1 Hz) increased an increase in [Ca\(^{2+}\)]\(_i\), in only 5.72 ± 1.44% of cells, a value not significantly different from that of cells during the no flow period (4.35 ± 1.31%) (p > 0.05) (Fig. 1C). However, within 30 s of starting oscillatory fluid flow with the media containing 2% FBS, 65.9 ± 3.94% of cells increased [Ca\(^{2+}\)]\(_i\), a value significantly greater than the no flow control (5.96 ± 1.05%) (p < 0.01) (Figs. 1, B and C). In the presence of both 2% FBS and apyrase (10 units/ml), the percentage of cells responding with an increase in [Ca\(^{2+}\)]\(_i\), was 3.91 ± 1.62%, a value not significantly different from the percentage responding during the no flow period (2.64 ± 1.98%) (p > 0.05). As was the case with our previous studies (9), the average amplitude of [Ca\(^{2+}\)]\(_i\) in all responding cells during all oscillatory fluid flow conditions was not significantly different from that of cells during no flow periods (data not shown).
FIG. 1. Oscillatory fluid flow induces increases in [Ca$^{2+}$]i in MC3T3-E1 osteoblastic cells. A, monolayers of MC3T3-E1 cells were loaded with fura-2 then exposed to oscillatory fluid flow (2N/m$^2$, 1Hz) during fluorescence ratio imaging. Times after stimulation in seconds is indicated on the top. The top three images are from cells exposed in MEM-$\alpha$, and the middle three images are from cells in MEM-$\alpha$+2%FBS. The bottom images are from cells in MEM-$\alpha$, 2%FBS in the presence of apyrase (10 units/ml). The pseudocolor map represents calibrated [Ca$^{2+}$]i from cells in MEM-$\alpha$, 2%FBS. In the presence of apyrase (10 units/ml), respectively, 1.23 and 2.28% of MC3T3-E1 cells responding with an increase in [Ca$^{2+}$]i, was not significantly different from that during no flow periods ($p > 0.05$). Each bar represents the mean ± S.E. of six individual experiments, and the three groups have a total of three experiments.

**A** To identify which purinoceptors are responsible for Ca$^{2+}$ mobilization, purinoceptor agonists or antagonists were added to the flow medium. In the presence of 2% FBS, pyridoxal phosphate 6-azophenyl-2',4'-disulfonic acid (PPADS) (30 μM), a P2X antagonist, did not have any effect on the Ca$^{2+}$ response relative to control. The presence of 2% FBS, pyridoxal phosphate 6-azophenyl-2',4'-disulfonic acid (PPADS) (30 μM), a P2X antagonist, did not have any effect on the flow-induced Ca$^{2+}$, response relative to control. The presence of 2% FBS, pyridoxal phosphate 6-azophenyl-2',4'-disulfonic acid (PPADS) (30 μM), a P2X antagonist, did not have any effect on the flow-induced Ca$^{2+}$, response relative to control.

**B** The graph depicts the percentage of MC3T3-E1 cells responding to oscillatory (Osc.) flow with an increase in [Ca$^{2+}$]i, was not significantly different from that during no flow periods ($p > 0.05$). Each bar represents the mean ± S.E., each group of data is obtained from six individual experiments, and the three groups have a total of 309, 334, and 298 cells, respectively.

**C** The graph depicts the percentage of MC3T3-E1 cells responding to oscillatory (Osc.) flow with an increase in [Ca$^{2+}$]i, was not significantly different from that during no flow periods ($p > 0.05$). Each bar represents the mean ± S.E., each group of data is obtained from six individual experiments, and the three groups have a total of 309, 334, and 298 cells, respectively.

ATP and UTP Are Involved in Fluid Flow-induced Ca$^{2+}$, Mobilization—To examine whether extracellular nucleotide triphosphates are involved in fluid flow-induced Ca$^{2+}$, mobilization, all five nucleotide triphosphates (ATP, UTP, GTP, CTP, and TTP) were added individually to the flow medium to replace 2% FBS. In the presence of 5 μM ATP or UTP, exposure to oscillatory fluid flow resulted in 74.3 ± 5.59% or 75.5 ± 2.28%, respectively, of cells displaying an increased [Ca$^{2+}$]i, whereas 1.19 ± 0.51% or 0.96 ± 0.57% of cells did so during no flow periods ($p < 0.01$). However, in the presence of 2% FBS in the presence of apyrase (10 units/ml), the percentage of MC3T3-E1 cells responding with an increase in [Ca$^{2+}$]i, was not significantly different from that during no flow periods ($p > 0.05$). Each bar represents the mean ± S.E., each group of data was obtained from six individual experiments, and the three groups have a total of 309, 334, and 298 cells, respectively.

**P2Y Purinoceptors Responsible for Ca$^{2+}$, Mobilization**

ATP and UTP, are able to restore the ability of oscillatory fluid flow to increase [Ca$^{2+}$]i, in the absence of FBS. The graph depicts the percentage of MC3T3-E1 cells responding to oscillatory flow (2N/m$^2$, 1Hz) with an increase in [Ca$^{2+}$]i, in MEM-$\alpha$ with ATP, UTP, GTP, CTP, or TTP added, individually, to replace 2% FBS. Only with added ATP or UTP was the percentage of cells responding to oscillatory (Osc.) flow with an increase in [Ca$^{2+}$]i, significantly different from that in the no flow period ($p < 0.01$). Each bar represents the mean ± S.E., and each experiment was repeated on 4–6 slides.
of the oscillatory fluid flow control (2% FBS) (*, p < 0.01). However, pertussis toxin (PTX; 200 ng/ml), which inhibits Gαs proteins, to which P2Y receptors but not other purinoceptors are coupled, dramatically reduced fluid flow-induced increases in [Ca2+]i (*, p < 0.01, versus flow control with 2% FBS). Each bar represents the mean ± S.E., and each experiment was repeated on 4–6 slides.

P2Y2 Purinoceptors Are Involved in Fluid Flow-induced Ca2+ Mobilization—Our agonist and antagonist results suggested that P2Y2 purinoceptors contribute to oscillatory fluid flow-induced increases in [Ca2+]i. Therefore, we examined the function of P2Y2 purinoceptors in MC3T3-E1 osteoblastic cells. The dependence of the rise in [Ca2+]i on ATP, UTP, ADP, and UDP concentration was assessed (Fig. 4), and the equipotency of ATP and UTP suggested P2Y2 purinoceptor expression in the MC3T3-E1 cells. To further confirm that P2Y2 purinoceptors are involved in fluid flow-induced Ca2+ mobilization, MC3T3-E1 cells were exposed to fluid flow in the presence of mouse P2Y2 antisense oligodeoxynucleotides. ATP (5 μM) increased [Ca2+], in P2Y2-SCR-treated MC3T3-E1 cells but did so to a lesser degree in P2Y2-AS-treated cells (74.9 ± 3.36 nM versus 136.4 ± 6.29 nM, respectively, p < 0.01) (Fig. 5A).

P2Y2-AS treatment significantly decreased the percentage of MC3T3-E1 cells responding to fluid flow with an increase in [Ca2+]i, relative to P2Y2-SCR-treated cells (29.0 ± 9.03% versus 65.3 ± 7.69%; p < 0.05) in the presence of 2% FBS (Fig. 5B). We next examined oscillatory fluid flow responsiveness in ROS 17/2.8 osteoblastic cells, which normally do not express P2Y2 purinoceptors, genetically engineered to express P2Y2 purinoceptors (24). Oscillatory fluid flow induced an increase in [Ca2+]i, in significantly fewer wild type ROS 17/2.8 cells than in P2Y2 purinoceptors expressing ROS 17/2.8 cells (25.80 ± 6.0 versus 69.0 ± 5.87%; p < 0.01) (Fig. 6).

DISCUSSION

Although it is clear mechanical loading plays an important role in bone remodeling at the tissue level, little is known about the cellular and molecular mechanisms of bone cell mechanotransduction (2, 3). Accumulating evidence suggests that extracellular fluid flow induced by mechanical loading is a potential physiological signal to trigger osteoblastic cell response to mechanical loading and may be a better candidate for transducing mechanical loads than other biophysical signals including substrate deformation and endogenous electric fields (6–8, 30). Recently, we demonstrated that oscillatory fluid flow, which mimics fluid flow induced by mechanical loading in vivo, is a potent bone cell activator and induces Ca2+ release and increases osteopontin gene expression via extracellular signal-regulated kinase 1/2 and p38 activation (9). Additionally the increased osteopontin expression is Ca2+-dependent. However, the molecular mechanisms of fluid flow-induced Ca2+ mobilization are still unknown. Our findings are the first direct experimental evidence demonstrating that oscillatory fluid flow and the extracellular signaling molecules ATP and UTP, interacting with their specific P2Y2 purinoceptors, play an important role in bone cell mechanotransduction.

Our data demonstrate that extracellular signaling molecules contribute to oscillatory fluid flow-induced Ca2+ mobilization since oscillatory fluid flow did not activate Ca2+ mobilization in the absence of the extracellular signaling molecules present in FBS. This is consistent with prior studies that demonstrate that FBS modulates the Ca2+ response of primary cultured bone cells to steady fluid flow (10). In the absence of FBS steady fluid flow only minimally and inconsistently induced Ca2+ mobilization in primary osteoblastic cells.

Several lines of evidence suggest that ATP is involved in mechanically induced Ca2+ mobilization. For instance, steady fluid flow modulates the Ca2+ response to ATP in vascular endothelial cells (31). Additionally, exposure of rat aortic smooth muscle cells to mechanical stretch (32) and rabbit endothelial cells (33) as well as human astrocytoma cells (34) to fluid flow stimulates the release of ATP. In skeletal tissue ATP induces osteoblastic cell Ca2+ mobilization (35), and hydrostatic pressure stimulates ATP release from the chondrocytes in pellet cultures. Finally, the addition of apyrase, which rapidly hydrolyzes 5’ nucleotide triphosphates to monophosphates, prevents mechanically induced Ca2+ wave propagation in polarized epithelia cultures (27). Taken together, these results...
suggest that extracellular nucleotides such as ATP may contribute to oscillatory fluid flow-induced Ca\(^{2+}\) mobilization in osteoblastic cells.

Our results also demonstrate that two of the five nucleotide triphosphates, ATP and UTP, are able to restore the ability of fluid flow to increase [Ca\(^{2+}\)], in the absence of FBS, suggesting that ATP and UTP may mediate the effect of fluid flow on [Ca\(^{2+}\)]. Previous studies demonstrate that exposure of endothelial cells to steady fluid flow in the presence of ATP induces Ca\(^{2+}\) mobilization (31, 36). Additionally, it has been demonstrated that UTP is involved in the intracellular Ca\(^{2+}\) wave propagation induced by mechanical stimulation in polarized epithelia (27). Thus, our data as well as other studies suggest that ATP and UTP are involved in mechanically induced Ca\(^{2+}\) mobilization.

In this study, we demonstrated that P2Y receptors (P2Y2 or P2Y4) may be involved in the Ca\(^{2+}\) response to fluid flow by adding different purinoceptor agonists and antagonists to the flow media. Furthermore, treatment with P2Y2 antisense oligodeoxynucleotides decreased the percentage of MC3T3-E1 osteoblastic cells that respond to fluid flow with an increase in [Ca\(^{2+}\)]. Additionally, ROS 17/2.8 cells, which do not normally express P2Y2 purinoceptors, transfected with P2Y2 purinoceptors responded to fluid flow in the presence of 2% FBS, confirming the involvement of P2Y2 purinoceptors. Taken together, our data suggest that P2Y2 purinoceptors are responsible for oscillatory fluid flow-induced Ca\(^{2+}\) mobilization in osteoblastic cells. Similarly, P2Y2 purinoceptors have been demonstrated to be involved in mechanically induced Ca\(^{2+}\) wave propagation in airway epithelia in vitro (27).

Although our antisense treatment of MC3T3-E1 cells significantly decreased the percentage of cells responding with an increase in [Ca\(^{2+}\)], 29.0 ± 9.03% of the cells still responded to oscillatory fluid flow. Furthermore, antisense treatment only partially inhibited ATP-induced Ca\(^{2+}\) mobilization. This suggests that P2Y2 purinoceptors were still being expressed after antisense treatment. Previous studies demonstrated that wild type ROS 17/2.8 cells, which lack P2Y2 purinoceptors, do not respond to steady fluid flow or ATP with an increase in [Ca\(^{2+}\)] (21, 37). On the other hand, we found that oscillatory fluid flow induced an increase in [Ca\(^{2+}\)], in a very low percentage of ROS 17/2.8 cells, just over 25.8% of the cells exposed. The difference of our study and others may be due to a different source of ROS 17/2.8 cells and culture environment. It may also suggest that the other P2 purinoceptors expressed in ROS 17/2.8 cells may contribute to fluid flow-induced Ca\(^{2+}\) responses.

Although our data suggest that P2Y2 purinoceptors are responsible for oscillatory fluid flow-induced Ca\(^{2+}\) mobilization in osteoblastic cells, other studies demonstrated that in human endothelial cells steady fluid flow in the presence of ATP activates Ca\(^{2+}\) influx via P2X purinoceptors (36). This suggests that either endothelial cells utilize different purinoceptors for mechanotransduction or that steady fluid flow utilizes a different mechanism than oscillatory fluid flow. This emphasizes the importance of using a mechanical stimulus appropriate for the cells being examined when trying to elucidate mechanotransduction pathways. Furthermore, that endothelial cells may utilize different purinoceptors in mechanotransduction than do osteoblastic cells suggests that osteoblastic mechanotransduction pathways can be targeted, e.g., in developing bone anabolic agents, without affecting endothelial cell mechanotransduction.

The physiological consequence of mechanically induced activation of the ATP/cytosolic Ca\(^{2+}\) pathway in osteoblastic cells is unclear. However, ATP, through an interaction with P2X purinoceptors, activates DNA synthesis in human osteoblastic MG-63 cells (23). Additionally, ATP, acting through P2Y purinoceptors, modulates mesenchymal cell differentiation in vitro (38). These observations together with data suggesting that mechanical signals, including fluid flow, increase the expression of markers of osteoblastic differentiation (6, 39–44) suggest that extracellular nucleotides may mediate the effect of mechanical signals, especially fluid flow, on bone cell differentiation.

Several limitations should be considered when interpreting our results. For instance, although our results strongly suggest that P2Y2 purinoceptors are involved in fluid flow-induced Ca\(^{2+}\) mobilization, it is also likely the other P2Y purinoceptors, for instance P2Y4 purinoceptors, may be involved in Ca\(^{2+}\) mobilization. Therefore, the comprehensive characterization of all P2Y subtypes of osteoblastic cells and their roles in bone cell mobilization.
mechanotransduction is under way in our laboratory. From this study, it is still not clear whether extracellular nucleotides (ATP, UTP alone, or both) are released by cells after the cells sense oscillatory fluid flow or whether these nucleotides already exist locally around cells and are activated by fluid flow to induce Ca\(^{2+}\) mobilization. Further investigation of the dynamics of the extracellular nucleotide release during fluid flow is necessary. Finally, our P2Y purinoceptor results were conducted in two different osteoblastic cell lines. Therefore, it is likely that the results can be extrapolated to other osteoblastic cell lines including primary cultures.

In summary, we have shown that extracellular nucleotides ATP and UTP are essential for oscillatory fluid flow-induced Ca\(^{2+}\) mobilization in osteoblastic cells in the presence of FBS. Furthermore, P2Y, specifically P2Y2, is the purinoceptor responsible for oscillatory fluid flow-induced Ca\(^{2+}\) mobilization. Taken together, these data suggest that oscillatory fluid flow acts through the interaction of the extracellular nucleotides ATP/UTP with P2Y (P2Y2 or P2Y4) purinoceptors to induce Ca\(^{2+}\) mobilization in osteoblastic cells. Our studies shed new light of the molecular mechanisms responsible for oscillatory fluid flow induced Ca\(^{2+}\) mobilization in osteoblastic cells. The understanding of molecular mechanisms of fluid-induced Ca\(^{2+}\) mobilization in osteoblastic cells will provide guidance in developing novel therapeutic approaches to various diseases such as disuse osteopenia and age-related osteoporosis.

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