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Full paper

Shear stress-induced Ca^{2+} elevation is mediated by autocrine-acting glutamate in osteoblastic MC3T3-E1 cells

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ABSTRACT

Mechanical loading is an important regulatory factor in bone homeostasis. Neurotransmitters, such as glutamate and ATP, are known to be released from osteoblasts, but their roles have been less studied. In this study, we investigated the role of transmitter release in mechanotransduction. To identify from where transmitters were released, focal fluid flow was applied to a single cell of MC3T3-E1, mouse calvaria-derived osteoblastic cell line, by using a glass micropipette. Intracellular Ca^{2+} elevation induced by the focal shear stress was eliminated by either $GdCl_3$, a mechanosensing channel inhibitor, or removal of extracellular Ca^{2+}. On the other hand, the focal shear stress-induced Ca^{2+} elevation was also significantly suppressed by inositol triphosphate receptor antagonist or vesicular release inhibitors. These results suggest that not only mechanosensitive channel-mediated Ca^{2+} influx but also some autocrine transmitters are involved in mechanotransduction. Additionally, glutamate receptor antagonists, but not ATP receptor antagonist, suppressed most of the focal shear stress-induced Ca^{2+} elevation. Therefore, it is suggested that glutamate is released from osteoblasts following the activation of mechanosensitive Ca^{2+} channels and acts in an autocrine manner. The glutamate release may have a significant role in the initial event of mechanotransduction in bone tissue.

1. Introduction

Bones contain osteocytes, osteoblasts and osteoclasts, and these cells communicate with each other. Bone mass is maintained by a balance between the activities of bone-forming osteoblasts and bone-resorbing osteoclasts. It is well known that mechanical loading is an important regulatory factor in bone homeostasis (1). To adapt to mechanical load, cortical and trabecular bones are remodeled. Thus, in general, bone mass is increased by mechanical loading and is decreased by unloading (2,3). Although these results are well known, the molecular mechanism underlying mechanoadaptation is still not well understood.

Mechanical loading to bone tissue induces cytoskeletal strain and fluid flow through the canalicular network (4). In vitro experiments demonstrated that both mechanical strain and shear stress caused by fluid flow increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in osteoblasts (5,6). The $[Ca^{2+}]_i$ elevation is considered as an initial cellular response induced by mechanical stimulation. It is generally considered that shear stress-induced $[Ca^{2+}]_i$ elevation is mediated by mechanosensitive and Ca^{2+}-permeable channels, while it is also reported that intracellular Ca^{2+} release via inositol 1,4,5-triphosphate (IP_3) receptor is involved in $[Ca^{2+}]_i$ elevation (7). Although a number of studies have suggested candidates for molecules involved in mechanically induced $[Ca^{2+}]_i$ elevation, the detailed mechanism has been unclear (8).

In osteoblasts, some transmitters such as ATP and glutamate are released upon exposure to various stimulations (9–12). Expression of the receptors of ATP, P2Y receptor family and P2X receptor family, and those of glutamate, AMPA receptor, kainate receptor, NMDA receptor and metabotropic glutamate receptor, has been

Abbreviations: AP-5, DL-2-amino-5-phosphonopentanoic acid; 2-APB, 2-aminoethoxydiphenylborate; ATP, adenosine triphosphate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DMSO, dimethyl sulfoxide; IP_3, inositol 1,4,5-triphosphate; MC3T3-E1, mouse calvaria-derived osteoblastic cell line; PI-PLC, phosphoinositide-phospholipase C; PLC, phospholipase C.

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reported in osteocytes, osteoblasts and osteoclasts (13–16). Activation of these receptors can lead to $[Ca^{2+}]_i$ elevation via either Ca^{2+} influx through Ca^{2+}-permeable channels or intracellular Ca^{2+} release through the Gq/phosphoinositide-phospholipase C (PI-PLC) pathway. Although a number of studies have suggested that both ATP and glutamate are involved in bone remodeling (17,18), the mechanism by which these transmitters are involved in mechanotransduction in bone remains less understood.

In the present study, we investigated whether the transmitter release is involved in shear stress-induced $[Ca^{2+}]_i$ elevation in mouse calvaria-derived osteoblastic cell line, MC3T3-E1 cells. In many preceding studies of shear stress, a flow chamber system was commonly used. In these studies, systemic shear stress induced $[Ca^{2+}]_i$ elevation in approximately 50–70% of cells (8,19,20). This suggested that there was a difference of mechanical force of the individual cells received. Additionally, it is difficult to identify the

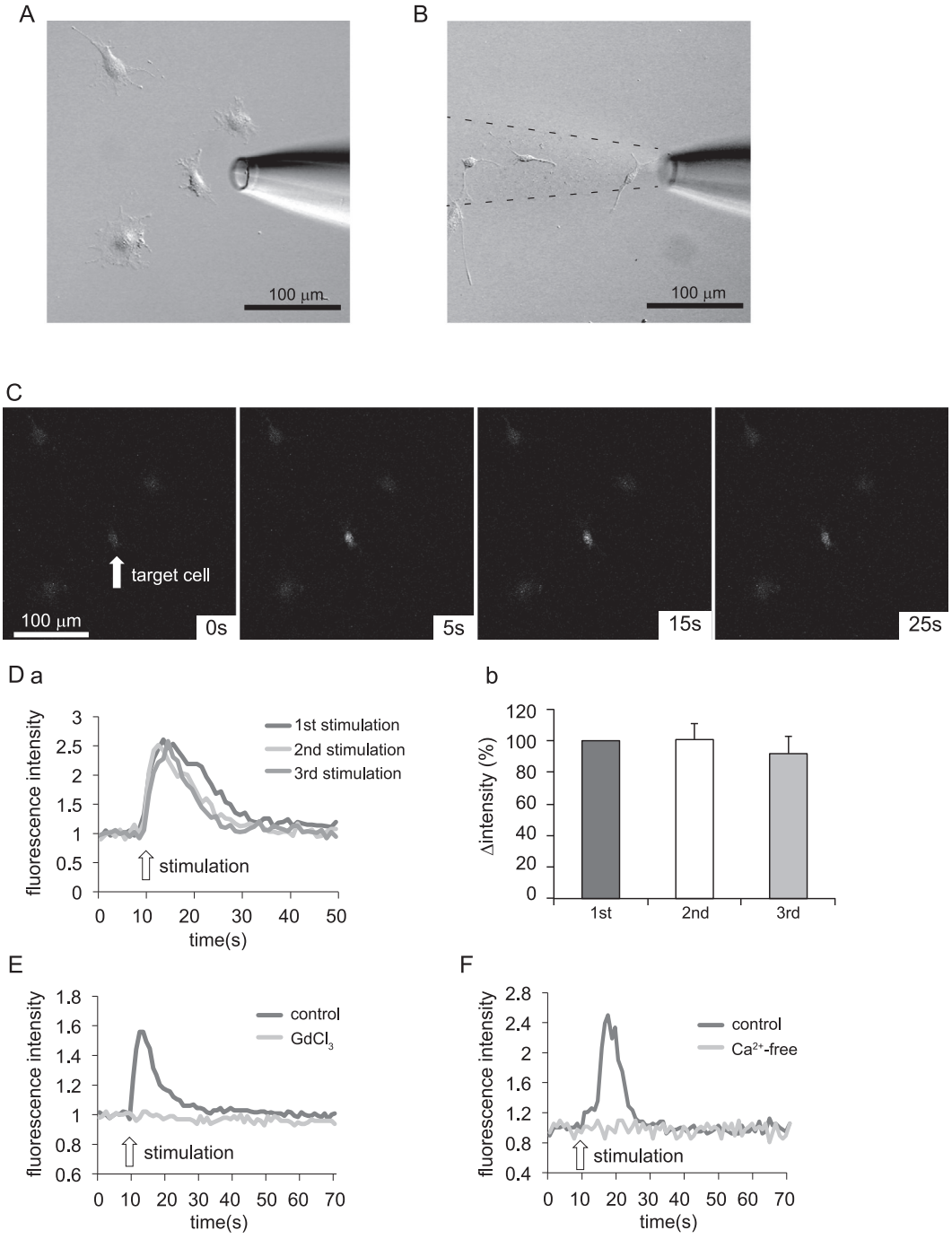

Fig. 1. Validation of focal fluid flow as mechanical stimulation. (A, B) A differential interference contrast microscopy image of a glass micropipette set close to the target cell (A) and fluid flow induced by air pressure (B). Broken lines indicate the area fluid was spread. (C) Fluo-3 fluorescence images just before and 5, 10 and 25 s after stimulation. An increase of fluorescence intensity was shown in the targeted cell, but not in the surrounding cells. (D) The $[Ca^{2+}]_i$ elevation induced by consecutive stimuli. Following recording of the baseline value, the focal fluid flow was applied via the micropipette (1 msec duration) and the interstimulus interval was 15 min. (a) Representative traces recorded from a single cell. (b) Average fold changes of fluorescence intensity in 2nd and 3rd stimulations compared with that in the 1st stimulation (n = 19). (E) The effect of $GdCl_3$ on focal fluid flow-induced $[Ca^{2+}]_i$ elevation. Representative traces of Ca^{2+} response in 1st stimulation (control, without $GdCl_3$) and 2nd stimulation (with 10 μM of $GdCl_3$). (F) Representative traces of Ca^{2+} response in normal and Ca^{2+}-free extracellular solution. Values are shown as the mean ± SEM.

cells releasing transmitters. Therefore, autocrine/paracrine or direct/indirect effects is indistinguishable in a flow chamber system. To study the significance of transmitter release from the osteoblasts that directly received mechanical stimulation, we used a method to apply focal and repeatable fluid flow by using a glass micropipette and a picospritzer. The focal shear stress-induced $[Ca^{2+}]_i$ elevation was suppressed by $GdCl_3$, a mechanosensing channel blocker, and also by vesicular release inhibitors. Moreover, glutamate receptor antagonists, but not ATP receptor antagonist, significantly suppressed the focal shear stress-induced $[Ca^{2+}]_i$ elevation. These results suggest that glutamate released by shear stress is involved in the primary response of mechanotransduction in osteoblasts.

2. Materials and methods

2.1. Cell culture

Cells of the mouse osteoblastic cell line MC3T3-E1 were purchased from Riken Cell Bank (Ibaraki, Japan). The cells were cultured in alpha-modified minimum essential medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Moregate Biotech, Bulimba, Australia), 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in 95% humidified air containing 5% CO_2. After several days of culture, they were seeded at very low density on a glass coverslip 1–2 days before the experiments for optical measurements of $[Ca^{2+}]_i$.

2.2. Optical measurements of $[Ca^{2+}]_i$

After 1–2 days of culture on a glass coverslip, MC3T3-E1 cells were loaded with 2.5 μM fluo-3AM, a Ca^{2+} fluorescent indicator, for 30 min and washed three times with normal extracellular solution, which contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 10 mM D-glucose (pH adjusted to 7.4 with NaOH), just before use. Then, the glass coverslip was transferred to a superfusion chamber on the stage of a confocal laser scanning microscope (LSM710, Carl Zeiss, Germany). Using a 10×/0.45 N.A. objective lens, the fluorescence was recorded for the excitation signal at 488 nm at 2 s intervals and analyzed with ZEN 2009 software (Carl Zeiss). Cells were superfused with normal extracellular solution or Ca^{2+}-free extracellular solution, which contained 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM D-glucose and 2 mM EGTA-4Na (pH adjusted to 7.4 with NaOH) at a rate of 2 ml/min. Unless otherwise noted, stock solutions of drugs were prepared and diluted 1000-fold into extracellular solution just before use and they were applied by bath application.

2.3. Application of focal shear stress

To apply focal shear stress, we used a glass micropipette and Pressure System IIe (Tohey Company, Fairfield, NJ, USA), which supplies repeatable air pressure pulses. The glass micropipettes were pulled from borosilicate glass capillaries with 1.5 mm outer diameter and 1.17 inner diameter (Harvard Apparatus, Edenbridge, UK) and had a tip diameter of approximately 30 μm. A glass micropipette filled with the extracellular solution was attached to the 3-axis micromanipulator, and then its tip was moved to about 30 μm from the target cell (Fig. 1A). The focal fluid flow was induced by air pressure (6.8 kPa, 1 msec duration) controlled by Pressure System IIe. The fluid was spread from the tip of a glass micropipette with about 100 μm width (Fig. 1B).

2.4. Chemicals

2-Aminoethoxydiphenylborate (2-APB), U73122, monensin, brefeldin A, suramin, 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt hydrate (CNQX), DL-2-amino-5-phosphonopentanoic acid (AP-5) and $GdCl_3$ were purchased from Sigma Aldrich (St. Louis, MO, USA). A Ca^{2+} fluorescent indicator, fluo-3AM, was purchased from Dojindo (Kumamoto, Japan). LY341495 was purchased from Tocris Biosciences (Bristol, UK). 2-APB, U73122, monensin, brefeldin A, CNQX and fluo-3AM were dissolved in DMSO. All other chemicals used were of reagent grade.

2.5. Statistical analysis

All data are expressed as mean ± SEM. In the optical measurements of $[Ca^{2+}]_i$, fluorescence intensity recorded from each cell was used for analysis. The amplitude of $[Ca^{2+}]_i$ elevation induced by shear stress was evaluated by comparing average of 10 points (20 s) of recording before the stimulus and average of 5 points (5 s) of recording from the peak of fluorescence intensity which appeared

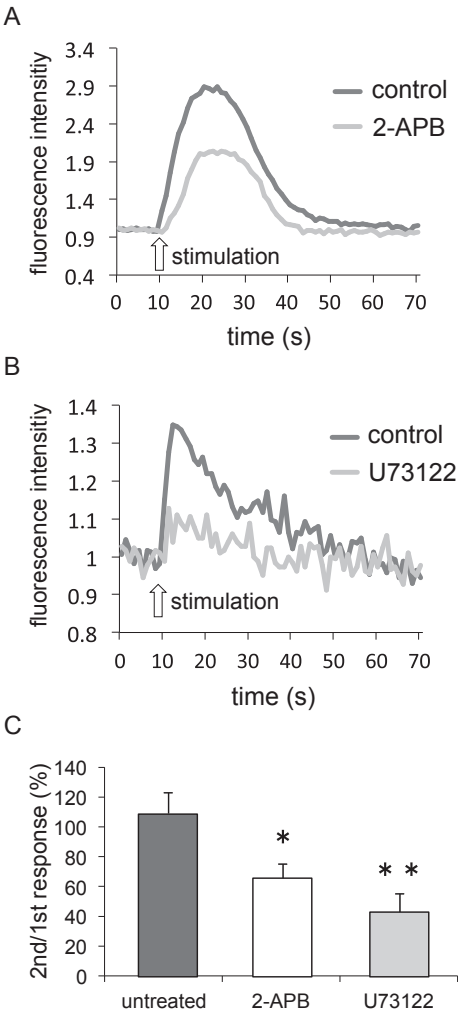

Fig. 2. Involvement of Gq/PI-PLC pathway in shear stress-induced $[Ca^{2+}]_i$ elevation. (A, B) Representative traces of Ca^{2+} response in the control and after treatment with 100 μM of 2-APB, an IP_3 receptor inhibitor (A), or 0.1 μM of U73122, a PLC inhibitor (B). (C) Ratios of average fold changes of fluorescence intensity recorded before and after treatment with drugs (untreated control, 2-APB and U73122, n = 20, 9 and 8, respectively). Values are shown as the mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$ compared with the control group.

after the stimulation. The data were recorded from more than 3 independent experiments. Student's *t*-test (two-tailed) was used to compare the data of 2 groups. The two-tailed *t*-test combined with Bonferroni's correction following one-way analysis of variance was used for multiple comparisons. Differences with p values <0.05 were considered significant.

3. Results

3.1. Validation of focal shear stress as mechanical stimulation

We first examined whether the focal fluid flow via a glass micropipette is appropriate as mechanical stimulation. The tip of a micropipette was set approximately 30 μm from a MC3T3-E1 cell, which was not attached to other cells (Fig. 1A). Application of 1 msec of focal fluid flow was sufficient to induce $[Ca^{2+}]_i$ elevation in all of the target cell. In contrast, no response was shown in the cells around the target cell by the stimulation (Fig. 1C). This result suggests that the fluid flow via the micropipette was controlled to a very narrow area. Additionally, this Ca^{2+} response was highly reproducible in the target cell (Fig. 1D). In the presence of 10 μM $GdCl_3$, an inhibitor of mechanosensitive and Ca^{2+}-permeable channels, the focal shear stress-induced $[Ca^{2+}]_i$ elevation was completely abolished (Fig. 1E). Additionally, by removal of Ca^{2+} from extracellular solution, the $[Ca^{2+}]_i$ elevation was also eliminated (Fig. 1F). Along with previous studies, our data suggest that the shear stress by focal fluid flow induced Ca^{2+} influx via mechanosensitive channels in MC3T3-E1 cells. However, the duration of $[Ca^{2+}]_i$ elevation was remarkably longer than that of the stimulus (Fig. 1D). Therefore, we considered that other pathways were involved in the shear stress-induced $[Ca^{2+}]_i$ elevation.

3.2. Involvement of exocytosis of transmitters and PI-PLC pathway in shear stress-induced $[Ca^{2+}]_i$ elevation

It has been also reported that Ca^{2+} release from endoplasmic reticulum is involved in mechanotransduction (10). Therefore, we examined whether the PI-PLC pathway was involved in the focal shear stress-induced $[Ca^{2+}]_i$ elevation. An IP_3 receptor antagonist, 2-APB, and an inhibitor of PLC, U73122, significantly suppressed the shear stress-induced $[Ca^{2+}]_i$ elevation (Fig. 2). Next, we examined whether vesicular exocytosis from a MC3T3-E1 cell is involved in

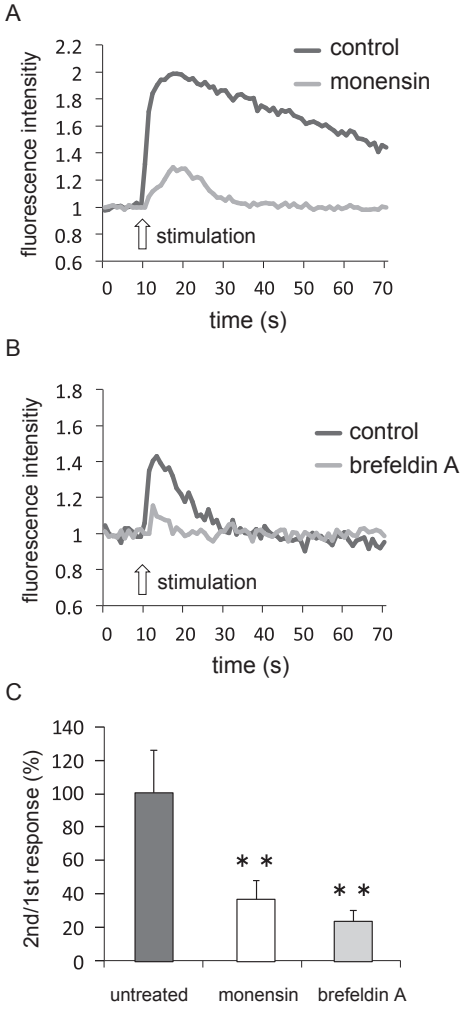

Fig. 3. Involvement of exocytosis of transmitters in shear stress-induced $[Ca^{2+}]_i$ elevation. (A, B) Representative traces of Ca^{2+} response in the control and after treatment with 100 μM of monensin (A) or 10 μM of brefeldin A (B). (C) Ratios of average fold changes of fluorescence intensity recorded before and after treatment with drugs (untreated control, monensin and brefeldin A, n = 14, 13 and 13, respectively). Values are shown as the mean ± SEM. $^{**}p < 0.01$ compared with the control group.

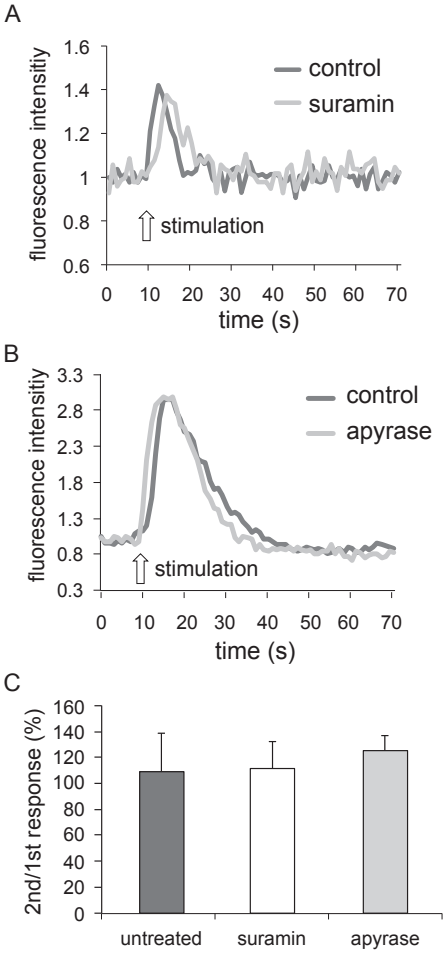

Fig. 4. The effects of P2 receptor inhibitor and ATP phosphatase on focal shear stress-induced $[Ca^{2+}]_i$ elevation. (A, B) Representative traces of Ca^{2+} response with and without 10 μM of suramin, a broad spectrum antagonist of P2X and P2Y receptor (A), or 5 U/ml of apyrase, an ATP phosphatase (B). (C) Ratios of average fold changes of fluorescence intensity recorded before and after treatment with drugs (untreated control, suramin and apyrase, n = 10, 9 and 13, respectively). Values are shown as the mean ± SEM.

the focal shear stress-induced $[Ca^{2+}]_i$ elevation by using monensin, which prevents vesicle formation from the Golgi apparatus, and brefeldin A, which causes disruption of the Golgi apparatus. Pretreatment with monensin at 100 μM for 30 min significantly suppressed the focal shear stress-induced $[Ca^{2+}]_i$ elevation. Similarly, the Ca^{2+} response was significantly suppressed by pretreatment with 10 μM brefeldin A for 30 min (Fig. 3). These results suggest that some transmitters were released from the mechanostimulated MC3T3-E1 cells by exocytosis and acted as an autocrine signal.

3.3. Identification of transmitter released from mechanostimulated cells

To identify the transmitter released from MC3T3-E1 cells upon exposure to focal shear stress, we examined whether ATP or glutamate was involved in the shear stress-induced $[Ca^{2+}]_i$ elevation by using suramin, a broad-spectrum antagonist of P2Y and P2X receptor, apyrase, which hydrolyzes extracellular ATP, LY341495, an antagonist of metabotropic glutamate receptor, CNQX, an antagonist of AMPA/kainate receptor, and AP-5, an antagonist of NMDA receptor. In the presence of suramin at 10 μM, the shear stress-induced $[Ca^{2+}]_i$ elevation was reproducible at a similar intensity. Similarly, 5 U/ml apyrase had no effect on the shear stress-induced $[Ca^{2+}]_i$ elevation (Fig. 4). We confirmed that both suramin and apyrase significantly suppressed bath-applied ATP-induced $[Ca^{2+}]_i$ elevation at the concentration used here (data not shown). In contrast, in the presence of LY341495 at 10 μM, the shear stress-induced $[Ca^{2+}]_i$ elevation was significantly suppressed. Similarly, 10 μM CNQX and 50 μM AP-5 also inhibited the Ca^{2+} response. Additionally, the combination of these glutamate receptor antagonists inhibited most part of shear stress-induced $[Ca^{2+}]_i$ elevation (Fig. 5). To rule out the possibility that glutamate receptor is directly activated by focal shear stress, we examined the effect of $GdCl_3$ on bath-applied glutamate-induced $[Ca^{2+}]_i$ elevation. Bath application

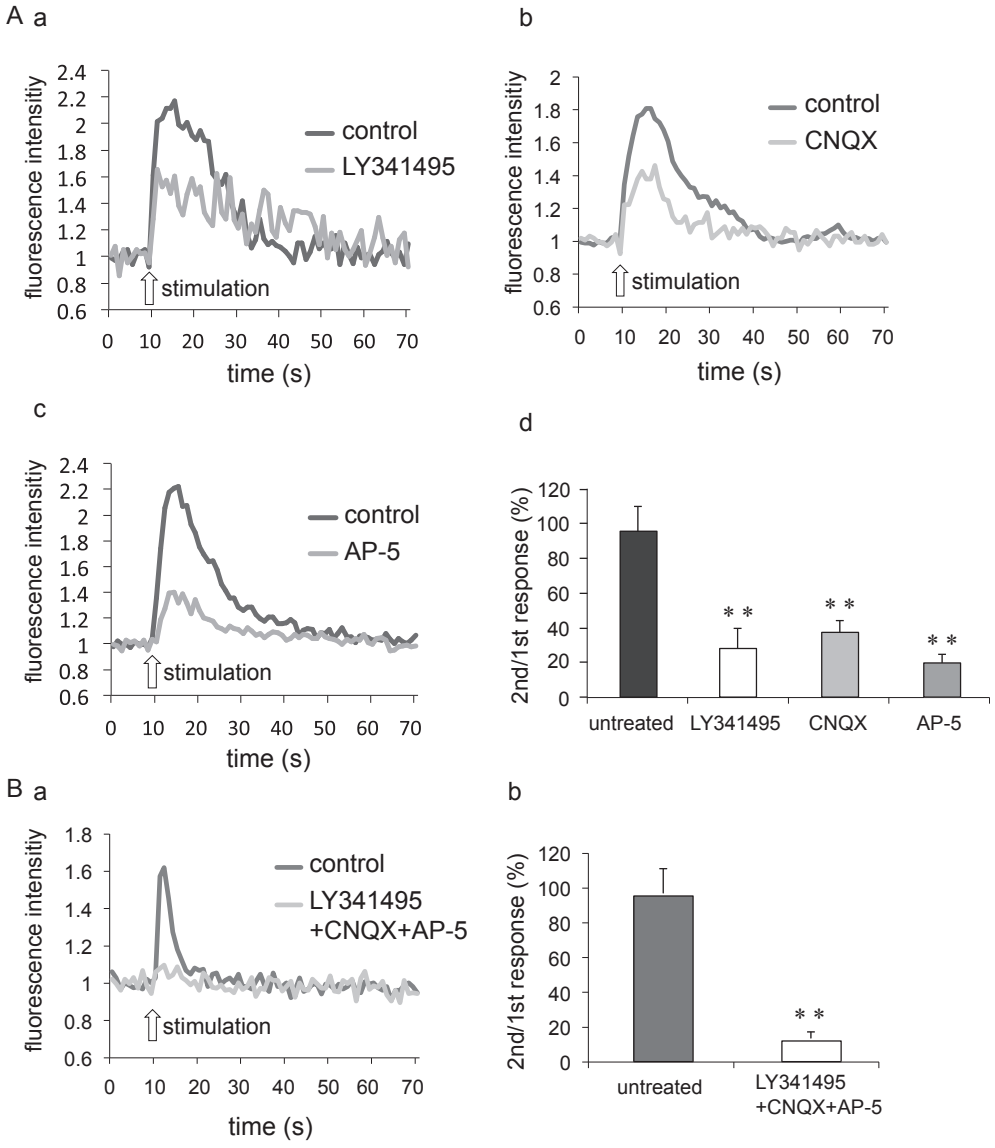

Fig. 5. Involvement of ionotropic and metabotropic glutamate receptors in focal shear stress-induced $[Ca^{2+}]_i$ elevation. (A) (a–c) Representative traces of Ca^{2+} response with and without 10 μM of LY341495 (a), 10 μM of CNQX (b) or 50 μM of AP-5 (c). (d) Summary of the effects of glutamate receptor antagonists on shear stress-induced $[Ca^{2+}]_i$ elevation (untreated control, LY341495, CNQX and AP-5, n = 10, 9, 10 and 10, respectively). (B) (a) Representative traces of Ca^{2+} response with and without combination of glutamate receptor antagonists. (b) Ratios of average fold changes of fluorescence intensity recorded before and after treatment with drugs (untreated control and glutamate receptor antagonist-treated, n = 12 and 11, respectively). Values are shown as the mean ± SEM. $^{**}p < 0.01$ compared with the control group.

of 1 mM glutamate increases $[Ca^{2+}]_i$ and the effect is significantly suppressed by LY341495, CNQX and AP-5, but not by $GdCl_3$ (Fig. 6). These results suggest that glutamate was released by focal shear stress and stimulated both metabotropic and ionotropic glutamate autoreceptors in MC3T3-E1 cells.

4. Discussion

It is generally believed that mechanical loading is one of the key regulators of bone mass. However, the detailed mechanism of mechanotransduction is less well understood. In the in vitro studies, various methods such as touch, hydrostatic pressure, stretch, fluid flow and vibration are known as mechanical stimulation, these are thought to induce deformation of cell membrane and activate mechanosensitive and Ca^{2+}-permeable channels (5,21–24). Although several ion channels such as L-type voltage-dependent Ca^{2+} channel, TRPV4 channel, connexin43 and TREK1 are suggested as candidates for mechanosensitive channels in osteoblasts, how much these molecules contribute to mechanosensing is unclear (3,6,25,26). In vitro studies demonstrated that both mechanical strain and fluid shear stress induced $[Ca^{2+}]_i$ elevation in osteoblasts (5,6). In an in vivo experiment, a strain level of approximately 1200 μstrain, which is several times greater than that during normal walking, was needed to elicit a significant osteogenic response in mouse tibia (2). In the in vitro study comparing mechanical strain and fluid shear stress, mechanical strain of less than 5000 μstrain was insufficient to induce cellular responses, such as the production of nitric oxide and prostaglandin E_2 in contrast, a physiological level of fluid flow (1.0 dyn/cm^2) was capable of inducing these responses (27). It suggests that shear stress induced by strain-derived flow of interstitial fluid is more important than mechanical strain itself in the mechanoadaptive response.

In the present study, by using the focal fluid flow system via a glass micropipette, we can apply fluid shear stress to a single cell directly and reproducibly. It allowed us to study the primary response of mechanotransduction. Application of 1 msec of focal fluid flow was sufficient to induce $[Ca^{2+}]_i$ elevation. Strong fluid shear stress induces decrease of reactivity to the following stimulations due to increase of actin stress fiber formation and cell stiffness in osteoblast (28). We can't calculate the mechanical force of the individual cells received. However, a decline of reactivity to the fluid flow used here was never shown in the target cells (Fig. 1D). It suggests the mechanical force received the cells was in physiological range.

The focal shear stress-induced $[Ca^{2+}]_i$ elevation was completely suppressed by $GdCl_3$ or removal of extracellular Ca^{2+} (Fig. 1). These results suggest that fluid shear stress induced Ca^{2+}-influx mediated by Gd-sensitive mechanosensitive channels. On the other hand, it is

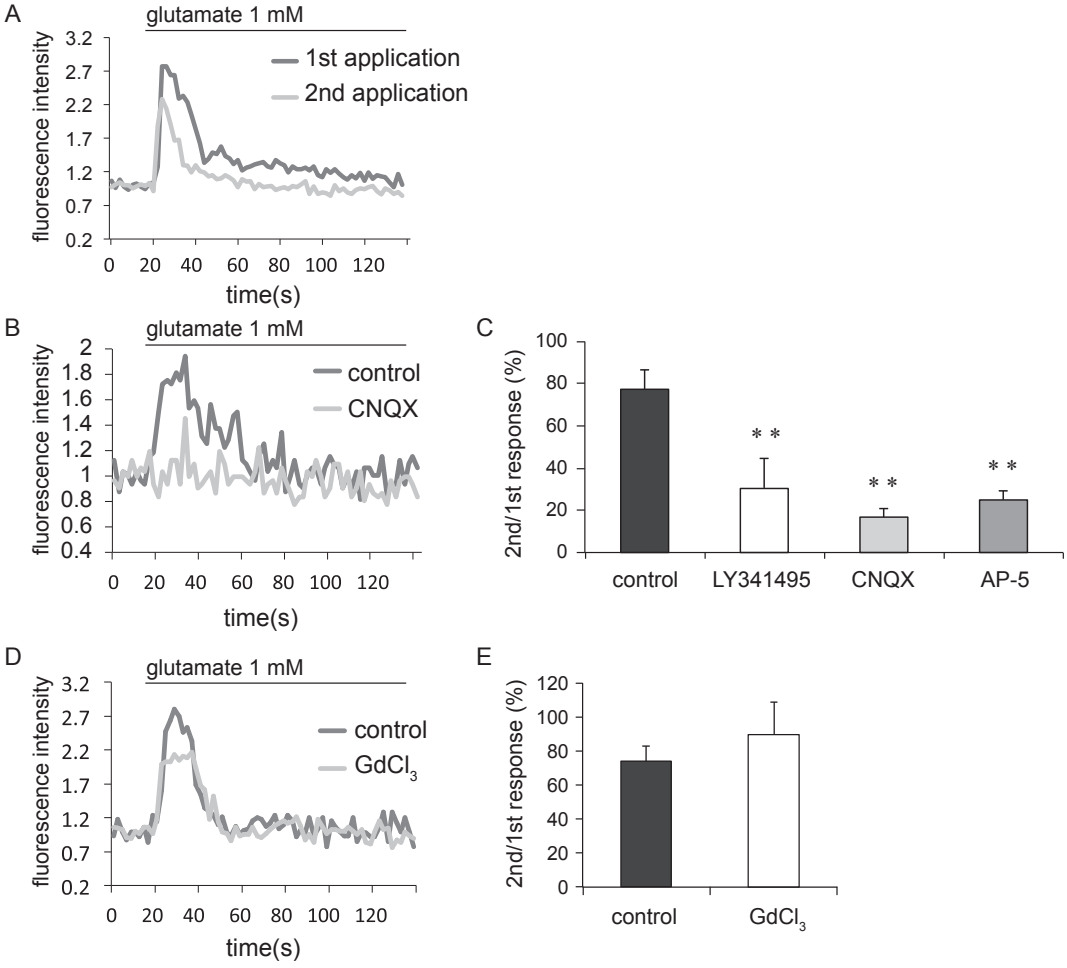

Fig. 6. The effects of $GdCl_3$ on bath-applied glutamate-induced $[Ca^{2+}]$ elevation. (A) Representative traces of $[Ca^{2+}]$ elevation induced by bath application of glutamate (1 mM). Glutamate was applied 2 times with a 15 min interval. (B) Representative traces of glutamate-induced Ca^{2+} response with and without 10 μM of CNQX. (C) Summary of the effects of glutamate receptor antagonists on bath-applied glutamate-induced $[Ca^{2+}]_i$ elevation (control, LY341495, CNQX and AP-5, n = 9, 5, 5 and 5, respectively). (D) Representative traces of glutamate-induced Ca^{2+} response with and without 10 μM of $GdCl_3$. (E) Summary of the effects of $GdCl_3$ on bath-applied glutamate-induced $[Ca^{2+}]_i$ elevation (control and $GdCl_3$, n = 5 and 5, respectively). Values are shown as the mean ± SEM. $^{**}p < 0.01$ compared with the control group.

also suggest that exocytosis and PI-PLC pathway are involved in shear stress-induced $[Ca^{2+}]_i$ elevation (Figs. 2 and 3). Furthermore, the $[Ca^{2+}]_i$ elevation was strongly inhibited by the combination of LY341495, CNQX and AP-5 (Fig. 5). The $[Ca^{2+}]_i$ elevation induced by bath application of glutamate was not affected by $GdCl_3$ (Fig. 6). These results suggest that Ca^{2+} influx via mechanosensitive channels acts as a trigger of exocytosis of glutamate from MC3T3-E1 cells and the released glutamate substantially contributes to the $[Ca^{2+}]_i$ elevation via ionotropic and metabotropic glutamate receptors. The glutamate-induced $[Ca^{2+}]_i$ elevation may cause further increase of $[Ca^{2+}]_i$ via downstream pathways (29–31). In the present study, the duration of fluid shear stress was 1 msec, nevertheless the $[Ca^{2+}]_i$ elevation was sustained for several seconds (Fig. 1D). Therefore, we considered that the translation from the Ca^{2+} influx via mechanosensitive channels to the signal via glutamate release is advantageous in terms of responding to instantaneous mechanical loading. Although extraordinary high levels of glutamate can induce oxidative toxicity during ischemia (32), the mechanism via transmitter release is thought to be unresponsive to highly consecutive stimuli. Thus, it is also useful to avoid excessive $[Ca^{2+}]_i$ elevation in physiological conditions.

Previous studies suggest that ATP is an important mediator of shear stress-induced cellular response in endothelial cells (20,33). Although it is also reported that ATP is released by fluid shear stress in osteoblasts (10), our data suggest that ATP was not involved in the initial response of osteoblasts to mechanical stimulation (Fig. 4). Lu et al. (34) have demonstrated that Ca^{2+} oscillation, but not first $[Ca^{2+}]_i$ elevation, induced by 9 min of fluid shear was suppressed by treatment with suramin in MC3T3-E1 cells. Gardinier et al. (28) have demonstrated that 12 dyn/cm^2 of fluid shear stress for 5 min increased actin stress fiber formation and enhanced cell stiffness via the $P2Y_2$ receptor in osteoblasts. They showed that knockdown of the $P2Y_2$ receptor suppressed shear stress-induced actin stress fiber formation; however, it had no effect on Ca^{2+} response to first stimulation. These studies suggest the possibility that ATP has protective roles against excessive or continuous loading. In general, ATP inhibits osteoblastic bone formation and stimulates bone resorption (17, 35). Thus, ATP is thought to be a negative regulator of bone mass. On the other hand, $P2X_7$ receptor knockout mice exhibited less sensitivity to mechanical loading-induced anabolic effect (36). Further studies are needed to reveal the role of ATP in mechanotransduction.

In general, glutamate is thought to be a positive regulator of bone mass. NMDA receptor antagonist, MK-801, was shown to decrease alkaline phosphatase activity and osteocalcin expression in primary osteoblasts (37). Chronic administration of either AMPA receptor antagonist or NMDA antagonist by osmotic minipump also reduced trabecular bone and cortical bone, respectively (38). Additionally, local injection of AMPA into the tibia increased bone volume in an in vivo experiment (39). Furthermore, pretreatment with NMDA enhanced mechanical strain-induced differentiation of osteoblasts (40). Our data presented in this study suggest that glutamate was released from MC3T3-E1 cells by shear stress and contributed substantially to the primary Ca^{2+} response to mechanical stimulation by acting in an autocrine manner. Therefore, there is the possibility that glutamate released from osteoblasts plays a vital role in mechanical loading-induced anabolic effect. It has been reported that in vivo mechanical loading decreased the expression of ionotropic glutamate receptors in osteoclasts and bone lining cells (41). Meanwhile, mechanical unloading also decreased NMDA receptor in disuse osteopenia (42). These effects on the expression of glutamate receptors may cause changes in the sensitivity to mechanical loading.

Although, in the present study, MC3T3-E1 cells were seeded at low density to avoid intercellular communication, so no response was shown in surrounding cells, it can be considered that glutamate released from an osteoblast induced by shear stress stimulates not only a mechanostimulated cell itself but also surrounding cells. Besides the facilitatory effects on osteoblast differentiation, glutamate also has suppressive effects on osteoclastic bone resorption (37–39, 43). Therefore, there is the possibility that shear stress-induced glutamate release contributes anabolic effects on bone mass by acting in both osteoblasts and osteoclasts.

In the present study, we demonstrated that glutamate was released from osteoblasts by shear stress and contributed substantially to shear stress-induced $[Ca^{2+}]_i$ elevation via the activation of both ionotropic and metabotropic glutamate receptors in an autocrine manner. It is suggested that glutamate plays a vital role in the primary response of mechanotransduction. These results will aid our understanding of the mechanism behind the regulation of bone metabolism by mechanical loading.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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