Prostaglandin E$_2$ modulates F-actin stress fiber in FSS stimulated MC3T3-E1 cells in a PKA dependent manner

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Running title: Effect of PGE$_2$ on F-actin remodeling in osteoblasts

Abstract

The effect of prostaglandin E$_2$ (PGE$_2$) on bone mass has been well established in vivo. Previous studies have showed that PGE$_2$ increases differentiation, proliferation, and regulates cell morphology through F-actin stress fiber in statically cultured osteoblasts. However, the effect of
PGE$_2$ on osteoblasts in the presence of fluid shear stress (FSS), which could better uncover the anabolic effect of PGE$_2$ in vivo, has yet to be examined. Here, we hypothesized that PGE$_2$ modulates F-actin stress fiber in FSS-stimulated MC3T3-E1 osteoblastic cells through PKA pathway. Furthermore, this PGE$_2$-induced F-actin remodeling was associated with the recovery of cellular mechanosensitivity. Our data showed that treatment with 10 nM dmPGE$_2$ for 15 min significantly suppressed the F-actin stress fiber intensity in FSS stimulated cells in a PKA dependent manner. In addition, dmPGE$_2$ treatment enhanced the cells’ calcium peak magnitude and the percentage of responding cells in the second FSS stimulation, though these effects were abolished and attenuated by co-treatment with phalloidin. Our results demonstrated that 10 nM dmPGE$_2$ was able to accelerate the “reset” process of F-actin stress fiber to its pre-stimulated level partially through PKA pathway, and thus promoted the recovery of cellular mechanosensitivity. Our finding provided a novel cellular mechanism by which PGE$_2$ increased bone formation as shown in vivo, suggesting that PGE$_2$ could be a potential target for treatments of bone formation related diseases.

**Keywords:** prostaglandin E$_2$; intracellular calcium; cytoskeleton; PKA pathway; fluid shear stress (FSS)
Introduction

Prostaglandin E$_2$ (PGE$_2$), a 20-carbon proinflammatory prostanoid, has various physiological effects on tissue. In particular, the anabolic effect of PGE$_2$ on bone has been established in vivo [1,2]. *In vitro* studies have showed that PGE$_2$ stimulates differentiation by elevating the intracellular cAMP level at low concentrations (~10$^{-8}$−10$^{-7}$ M), while stimulates proliferation through phosphatidylinositol turning over at high concentrations (~10$^{-6}$−10$^{-5}$ M) in statically cultured MC3T3-E1 osteoblastic cells [3-6]. However, during daily activities, bone cells are constantly stimulated by mechanical force, and the presence of mechanical force alters the release of biochemical factors [7,8] and the involvements of signaling pathways [9,10]. Thus, the cellular effect of PGE$_2$ in the presence of mechanical loading could be different from that in the static condition, which worth to be studied.

It is well accepted that mechanical loading in bone is anabolic in tissue and at cellular levels. Fluid shear stress (FSS), as one kind of common mechanical stimulation in bone [11], deformed the cell body, thus introducing rapid increase in intracellular calcium concentration ([Ca$^{2+}$]$_i$) [12] and the release of adenosine triphosphate (ATP) and PGE$_2$ [8] in osteoblasts. However, the mechanosensitivity of osteoblasts was found to be decreased during continuous FSS stimulation, but recovered after inserting a rest period [13]. The cytoskeleton, a mechanotransducer during FSS stimulation, was believed to play a central role in the regulation of cellular mechanosensitivity through the modulation of mechanosensitive calcium channel (MSCC) activity [14,15]. While PGE$_2$ has been shown to be capable of affecting cell morphology through modulating F-actin stress fiber in statically cultured osteoblasts [16] and also capable of promoting osteogenic differentiation in rat tendon stem cells [17], the PGE2-regulated F-actin stress fiber remodeling in the presence of mechanical stimulation and its effect on cellular mechanosensitivity have yet to be
addressed.

Protein kinase A (PKA) pathway is involved in mechanosensitivity regulation in bone cells. Activation of PKA has been shown to enhance the intracellular calcium response during FSS stimulation [18] and is involved in both FSS-induced ERK activation [19] and cyclooxygenase-2 up-regulation [20]. Furthermore, PKA-regulated RhoA GTPase suppression has also been demonstrated previously [21], suggesting that PKA is capable of modulating F-actin stress fiber [14,18]. Moreover, PGE₂ triggers intracellular cAMP elevation in MC3T3-E1 osteoblastic cells at low concentrations [3]. These data suggested that there was a connection between PKA and PGE₂-regulated F-actin remodeling in osteoblasts.

In the present study, we hypothesized that PGE₂ modulated F-actin stress fiber in FSS-stimulated MC3T3-E1 osteoblastic cells through PKA pathway and that this PGE₂-induced F-actin remodeling was associated with the recovery of cellular mechanosensitivity. To test this hypothesis, cells were treated with dmPGE₂ post FSS stimulation; the involvement of PKA pathways on F-actin modulation was investigated by using 8-Br-cAMP/PKI. To test the effect of dmPGE₂-induced F-actin stress fiber remodeling on the recovery of cellular mechanosensitivity, cells were treated with vehicle, dmPGE₂, and dmPGE₂ plus phalloidin between two bouts of FSS stimulations. The FSS-induced [Ca²⁺]ᵢ transient was used as an indicator of cellular mechanosensitivity.

Materials and Methods

Materials

16,16-dimethyl prostaglandin-E₂ (dmPGE₂; Cayman, Ann Arbor, USA) was used in this study due to its prolonged half life and bioactivities similar to PGE₂ [22]. Based on the finding that PGE₂
inhibites DNA synthesis by stimulating differentiation at low concentrations (~10^-8–10^-7 M) in MC3T3-E1 cells [3,4], dmPGE2 was used at a final concentration of 10 nM. Phalloidin (Sigma, St Louis, USA), an actin stabilizer, was used at a final concentration of 1 μM. 8-Bromoadenosine 3’, 5’-cyclic monophosphate sodium salt (8-Br-cAMP, Sigma), an agonist of PKA pathway, was dissolved in distilled water at a stock concentration of 15 mg/ml and used at a final concentration of 100 μM. The cAMP dependent protein kinase peptide inhibitor (PKI; Promega, Madison, USA), an inhibitor of PKA pathway, was used at a final concentration of 10 μM. All the final solutions were prepared with the flow medium that was used in F-actin staining and calcium imaging studies. The concentration of agonist or inhibitor was chosen based on our previous studies [14,23].

**Cell culture**

MC3T3-E1 cells were purchased from American Type Culture Collection (ATCC; Manassas, USA). Cells were grown in α-MEM (Sigma) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, USA) and 1% penicillin G and streptomycin (Sigma) and maintained in a humidified incubator at 37°C with 5% CO₂. Cells were seeded onto type-I collagen coated glass slides at an initial density of 4×10⁴ cells/cm² and used for calcium imaging and cytoskeleton staining studies once reaching 80% of confluence. Cells were serum starved overnight prior to each experiment. Cells of 3–10 passages were used in our study.

**F-actin staining**

The slide containing MC3T3-E1 cells was mounted to a custom designed parallel plate flow chamber [24] and subject to 20 min of FSS at 12 dyn/cm² by using aerated (5% CO₂/95% air) flow medium (α-MEM supplemented with 0.1% FBS, v/v) in a cell incubator (37°C). After FSS loading,
cells were treated with dmPGE₂, dmPGE₂ plus phalloidin, dmPGE₂ plus PKI, 8-Br-cAMP, 8-Br-cAMP plus PKI and vehicle (α-MEM supplemented with 0.1% FBS, v/v) for 15 min, respectively (Fig. 1A). After all treatments, cells were immediately washed with ice cold PBS, fixed with 2% paraformaldehyde and 0.1% triton X-100, and stained with Alexa Fluor 488 phalloidin (1:40 dilution; Invitrogen, Grand Island, USA) and DRAQ5™ (1:2000 dilution; Biostatus Ltd., Leicestershire, UK). Stained cells were imaged on an inverted confocal laser scanning microscope (Zesis LSM 510; Heidenheim, Germany) using 488 nm and 631 nm excitations with a 40× objective. Since the F-actins network is a 3D structure, to minimize the variation among different batches, we thus recorded our images by focusing the z-level of most nucleuses in a random view. Each experiment was repeated thrice and at least 9 image fields of 40× magnitude were obtained in each trial.

**Image quantification**

Volocity software (version 4.3; Improvision, Waltham, USA) was used to process the areal fraction of continuous F-actin stress fibers in individual cells from well documented confocal images (Supplementary Fig. S1). To account for the variations of staining process and laser intensity, which were unavoidably present among different batches of tests, we obtained a correction factor based on the nuclei staining intensity, since the nucleus was supposed to have a relatively constant intensity. This correction factor was used to linearly adjust the actin staining in each image. The continuous F-actin stress fibers were identified by setting two thresholds: objective intensity larger than 60, and an objective size larger than 500 pixels. The total area was obtained by setting a much lower threshold (larger than 7) and using the “fill holes in objects” option. The areal fraction of the continuous F-actin stress fibers was calculated, with the total areal of continuous F-actin stress
fibers being divided by the total area of cell. For each experimental group, three slides and 16 to 36 cells were processed.

**Calcium imaging**

Cells were washed with phosphate buffered saline (PBS), stained with 12 μg/ml Fluo-4 AM (Invitrogen) in α-MEM for 45 min at 37°C, and then washed with PBS again to remove the dye. The slide was mounted to a custom-designed parallel plate flow chamber [24], and fixed to the stage of a fluorescent microscope (Leica Microsystems, Wetzlar, Germany). Flow medium (α-MEM supplemented with 2% FBS, v/v), preserved at 37°C, was slowly driven into the chamber by a peristaltic pump (Longer Peristaltic Pump). The higher concentration of FBS (2%) in calcium imaging than that in F-actin staining study (0.1%) was used to ensure attachment and growth of MC3T3-E1 during the whole experimental process. After resting for 30 min, dynamic fluorescence intensity inside a randomly selected field (1431×1059 microns in dimensions) was recorded by real time [Ca^{2+}]_i imaging with a 20× objective at room temperature. The recording time course included a 60-s baseline, followed by two sequential FSS sessions (3 min each at 12 dyn/cm²) with a 15 min rest period inserted in between, during which dmPGE₂, dmPGE₂ plus phalloidin or vehicle (α-MEM supplemented with 2%FBS, v/v) was added to flow medium to test the effect of dmPGE₂ on the calcium response recovery and the involvement of F-actin intensity in second FSS (**Fig. 1B**). Image J (Version 1.44p, http://rsb.info.nih.gov/ij/) was used to analyze the fluorescence intensity in individual cells, which was normalized with the mean background intensity obtained in three randomly chosen blank areas. The calcium peak magnitude was reported as the fold increment of the peak intensity over the mean baseline fluorescence intensity (=1). The percentage of responding cells was determined for each test (the number of cells whose
calcium peak magnitude was over 1.25 fold of baseline was divided by the number of total cells). The 1.25-fold baseline threshold was chosen based on our previous study [12] using Fluo-2 under 12 dyn/cm², in which MC3T3-E1 cells remained the same, with regards to the ability of reacting with FSS (percentage of responding cells). Each experiment was repeated thrice and 47–102 cells per group were processed.

**Statistical analysis**

Data were presented as mean±SD. For the cytoskeleton image quantification data, statistical significance was determined by using one way ANOVA with Bonferroni’s post hoc test. A $P$-value $<0.05$ indicated statistical significance in all analyses. For calcium imaging data, the statistical significance of the calcium peak magnitude and the percentage of responding cells in all treatments were determined by a one way ANOVA with Bonferroni’s post hoc test and chi-square test, respectively.

**Results**

**Effects of dmPGE$_2$ and PKA pathway on F-actin intensity**

When compared with FSS plus vehicle treatment group (Fig. 2A, b), dmPGE$_2$ treatment after FSS stimulation significantly decreased the FSS-induced F-actin stress fiber intensity (Fig. 2A, c) to nearly pre FSS status (Fig. 2A, a). Co-treated cells with dmPGE$_2$ plus phalloidin (Fig. 2A, d) or dmPGE$_2$ plus PKI (Fig. 2C, a) caused an impaired inhibitory effect of dmPGE$_2$. Similar to dmPGE$_2$ post treatment, 8-Br-cAMP suppressed the F-actin stress fiber intensity in FSS-stimulated cells (Fig. 2C, b), which was again abolished by co-treatment with PKI (Fig. 2C, c).
Quantification of the areal fractions of F-actin stress fibers

Under the present imaging and threshold settings, the areal fraction of continuous F-actin stress fiber in pre FSS group was 0.28±0.05, and was significantly increased to 0.79±0.03 by FSS plus vehicle treatment. dmPGE₂ significantly suppressed the FSS-induced increment in F-actin stress fiber intensity (0.32±0.05), which was comparable to the static level. Co-incubation of cells with phalloidin significantly attenuated the inhibition of dmPGE₂ (0.56±0.03). Similarly, co-treatment with PKI significantly decreased the inhibition of dmPGE₂ (0.6±0.03). The 8-Br-cAMP produced similar suppression as dmPGE₂ (0.45±0.05) and was completely inhibited by PKI (0.78±0.04) (Fig. 2B,D).

Effects of dmPGE₂ on the recovery of mechanosensitivity in MC3T3-E1 cells

Cells responded to the first FSS stimulation with a rapid increase in [Ca²⁺]ᵢ in all groups (Fig. 3, 1ˢᵗ FSS). In detail, the average [Ca²⁺]ᵢ peak magnitude and percentage of responding cells were 1.68±0.11 and 77.7±11.6% (52 of 67 cells) in the vehicle group, 1.63±0.07 and 71.2±19.9% (73 of 102 cells) in the dmPGE₂ treated group, and 1.62±0.06 and 68.3±7.5% (36 of 51 cells) in the dmPGE₂ plus phalloidin group; no statistical difference was found among these groups. However, it was found that dmPGE₂ treatment, post the first FSS, significantly increased the cells’ calcium peak magnitude and the percentage of responding cells in the second FSS stimulation by 1.25- and 5.31-fold, when compared with the vehicle treatment, which was respectively abolished and significantly attenuated by co-treating with phalloidin (Fig. 3, 2ⁿᵈ FSS). In detail, the average calcium peak magnitude and percentage of responding cells were 1.26±0.08 and 16.3±4.8% (8 of 47 cells) in the vehicle group, 1.57±0.37 and 86.5±9.3% (58 of 67 cells) in the dmPGE₂-treated
group, and 1.28±0.02 and 45.7±12.9% (32 of 69 cells) in the dmPGE\(_2\) plus phalloidin group.

**Discussion**

Mechanical forces and the downstream biochemical factors are important regulators for the maintenance of skeletal structure and mass. Osteoblasts respond to FSS with a [Ca\(^{2+}\)]\(_i\) transient and a quick burst of ATP release (between 1-5 min post FSS), followed by a delayed, but sustained, PGE\(_2\) release [8]. Previous studies have demonstrated a significant role of PGE\(_2\) in osteoblasts' differentiation and proliferation [3-6]. Results from the present study suggest a novel biological significance of PGE\(_2\), which might counteract with early released adaptation-promoting factors, such as ATP. The initially released ATP has been shown to stiffen cells by increasing the polymerization of actin filaments, and thus, promote the adaptation of osteoblasts to their mechanical environment [8]. However, this increased cellular stiffness also caused the loss of mechanosensitivity by decreasing the FSS-induced cellular deformation and opening the possibility of iron channels [14,25]. One major effect of exogenous PGE\(_2\), as derived from this study, is the resultant restoration of mechanosensitivity in osteoblasts, by way of softening cells through modulating F-actin stress fiber intensity post FSS stimulation. However, without the additional exogenous PGE\(_2\), the effect of released ATP overrode the effect of PGE\(_2\) on F-actin stress fiber due to the insufficient endogenous PGE\(_2\) release (around 10\(^{-10}\)–10\(^{-9}\) M, induced by 20 min of 30 dyn/cm\(^2\) FSS loading in MC3T3-E1 cells) [26], which was evidenced by fact that actin polymerization was still observed in vehicle group ([Fig. 2A, b](#Fig2A.b)).

A reciprocal regulation mechanism existed between FSS-induced [Ca\(^{2+}\)]\(_i\) transient and cytoskeleton remodeling. On one hand, as the earliest respondent upon receiving mechanical loading, [Ca\(^{2+}\)]\(_i\) transient was thought to be responsible for the subsequent release of ATP and
PGE₂, an alteration in gene expression [27] and cytoskeleton remodeling [28] in osteoblasts due to Ca²⁺ influx through the MSCC and voltage-sensitive Ca²⁺ channels (L-VSCC), as well as the intracellular Ca²⁺ release from endoplasmic reticulum (ER) [29]. On the other hand, treatments of cytochalasin D or parathyroid hormone disrupted/decreased F-actin stress fiber [14] increased the calcium response induced by FSS by decreasing the cellular stiffness, and modulating the open possibility of MSCC. Both sides suggested a direct connection between F-actin stress fiber and FSS induced calcium response. In our study, we found that dmPGE₂ treatment increased the [Ca²⁺]ᵢ transient during sequential FSS loading, which was abolished or attenuated by phalloidin. Our imaging quantification results showed that dmPGE₂ decreased the F-actin stress fiber intensity, while vehicle treatment increased it. These data together showed the participation of F-actin stress fiber in dmPGE₂ regulated mechanosensitivity in FSS-stimulated MC3T3-E1 cells. In addition, some cells in dmPGE₂ and phalloidin co-treated group showed delayed calcium peak (Fig. 3A, 2nd FSS), which suggested that phalloidin treatment caused the alteration of F-actin dynamics which might also be involved in mechanosensitivity in MC3T3-E1 cells.

Based on the observations that c-AMP/PKI respectively mimicked/attenuated the effect of PGE₂ on F-actin remodeling, we conclude that the PKA pathway is involved in PGE₂-induced F-actin remodeling. PGE₂ acts on osteoblasts through E-prostanoid (EP) receptors, in which EP1, EP2 and EP4 receptors are expressed in MC3T3-E1 cells [30]. EP2 and EP4 receptors are linked to Gs alpha subunit (Gs) to increase intracellular c-AMP level and activate PKA pathway [31]. Our preliminary data showed that PKA activation was able to augment the hypotonic stress induced [Ca²⁺]ᵢ transient in MC3T3-E1 cells (Supplementary Fig. S2A,B), suggesting a potential role of PKA in PGE₂ induced mechanosensitivity recovery in osteoblasts. We speculated that when osteoblasts are stimulated by FSS, the released and exogenous PGE₂ might act through either or
both EP2 and EP4 to activate PKA pathway and thus down-regulate F-actin intensity, eventually leading to the recovery of $[\text{Ca}^{2+}]_i$ transient (Fig. 4). Combined with previous reports showing that PKA pathway is involved in low dose PGE$_2$ induced cell differentiation in osteoblasts [3,4], our and others' data suggested that PGE$_2$ might facilitate osteoblasts to sense the FSS stimulation, thus amplifying the anabolic effect of FSS by promoting osteogenic differentiation. Our data also suggested that other signaling pathways might synergistically work with the PKA pathway, since PKA inhibition did not entirely blocked the effect of dmPGE$_2$ and c-AMP treatment was found not as effective as dmPGE$_2$ treatment.

In the present study, a novel quantification method was used to measure the difference among different treatment groups in F-actin stress fiber. This quantification method has several strengths. First, the fluorescent intensity fluctuation among different batches of tests was unavoidable and exhibited a great impact on the accuracy of quantification results. We thus obtained a correction factor based on the nuclei staining intensity to limit the influence. Second, FSS-induced actin polymerization increased F-actin stress fiber thickness and length [8], causing an elongated cell morphology [32]. By using double thresholds defining system in our method, we were able to detect the area fraction of continuous F-actin stress fiber changes cell by cell between different treatment groups, which provided a better description about the F-actin stress fiber intensity than the total intensity ratio measurement used in previous studies [9,10]. However, there’s still limitation in our study. The subtype(s) of EP receptor involved in dmPGE$_2$-modulated F-actin remodeling was not clear, since both EP2 and EP4 subtypes have been found to activate PKA pathway in different cell types [31, 33,34]. In addition, to better describe the mechanism of PGE$_2$ modulated mechanosensitivity recovery, the involvement of the PKA pathway in PGE$_2$ modulated calcium response and RhoA activation, as well as the long term effects of PGE$_2$ on mechanical
loaded cells, needs to be further studied.

In conclusion, our study demonstrated that 10 nM dmPGE$_2$ was able to accelerate the “reset” process of F-actin stress fiber to its pre-stimulated level partially through PKA pathway, thus promoting the recovery of cellular mechanosensitivity. Our finding provided a cellular mechanism by which PGE$_2$ increased bone formation in vivo, suggesting that PGE$_2$ could be a potential target for treatments of bone formation related diseases.

**Funding**

This work was supported by grants from the NIH (AR054385 and P20RR016458, DK058246, and AR051901), the National Natural Science Foundation of China (11272366, 11172207, and 10972243), the Visiting Scholar Foundation of Key Laboratory of Biorheological Science and Technology of Chongqing University, the Ministry of Education (No. CQKLBST-2012-002).

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**Figure legends**

**Figure 1. Experimental designs** (A) The time course of F-actin staining. MC3T3-E1 cells were subject to FSS stimulation (12 dyn/cm^2) for 20 min, then treated with dmPGE_2 (10 nM), dmPGE_2 (10 nM) + phalloidin (Phall, 1 μM), vehicle, dmPGE_2 (10 nM)+PKA inhibitor (PKI, 10 μM), PKA activator (8-Br-cAMP, 100 μM), and PKI (10 μM)+8-Br-cAMP (100 μM) for 15 min in
static. F-actin was immediately stained after all treatments. (B) The time course of intracellular calcium imaging. MC3T3-E1 cells were subjected to two bouts of FSS stimulation (3 min at 12 dyn/cm²), between which cells were treated with dmPGE₂ (10 nM), dmPGE₂ (10 nM)+phalloidin (PHALL, 1 μM) or vehicle for 15 min in static. The intracellular calcium response was recorded during the two FSS loading processes.

Figure 2. Involvement of PKA in dmPGE₂-modulated F-actin remodeling

(A) Representative F-actin and nuclei staining showing effect of dmPGE₂ on F-actin stress fiber intensity in MC3T3-E1 cells (a–d). (B) Quantification results of (A). Compared with Pre-FSS, FSS+vehicle treatment significantly increased the F-actin stress fiber intensity, which was greatly decreased by FSS+dmPGE₂. Phalloidin (Phall) significantly attenuated the effect of dmPGE₂. (C) Representative F-actin and nuclei staining showing the involvement of PKA in dmPGE₂-modulated F-actin stress fiber intensity (a–c). (D) Quantification results of (C). The effect of dmPGE₂ was significantly attenuated by PKI. 8-Br-cAMP produced similar effect as dmPGE₂ which was blocked by co-treating with PKI. *P<0.05 vs. FSS+vehicle group, †P<0.05 vs. Pre-FSS group, ‡P<0.05 vs. FSS+dmPGE₂ group, §P<0.05 vs. FSS+8-Br-cAMP group.

Figure 3. dmPGE₂ modulates mechanosensitivity recovery

(A) Representative traces of [Ca^{2+}]_i responses during two bouts of FSS stimulation. (B) The calcium peak magnitude (baseline=1) and (C) percentage of responding cells during two bouts of FSS stimulations. During the second FSS, the vehicle-treated cells showed reduced calcium peak-magnitude and the percentage of responding cells in the second FSS stimulation. In contrast, compared to first FSS, 10 nM PGE₂ treated cells showed no significant difference in calcium peak magnitude and an
increased percentage of responding cells. Phalloidin (Phall) abolished the recovery of calcium peak magnitude and significantly attenuated the recovery of responding cells induced by dmPGE₂. Each experimental group was repeated thrice (n=3 in C), 47−102 cells were used for data analysis (B). aP<0.05 vs. its own 1ˢᵗ FSS, bP<0.05 vs. 2ⁿᵈ FSS in dmPGE₂ group, cP<0.05 vs. 2ⁿᵈ FSS in vehicle group.

**Figure 4. Proposed working model of extracellular PGE₂ on mechanosensitivity ([Ca²⁺]ᵢ) of FSS-stimulated MC3T3-E1 cells**  PGE₂ acted on EP receptor (GRCP), followed by release of Gs alpha subunit (Gs), cAMP elevation [as a consequence of the activation of adenylate cyclase (AC)], and PKA activation. The activated PKA pathway might suppress F-actin polymerization and cause the recovery of calcium response. Dash line, transmembrane; solid line, intramembrane.

**Supplementary Figure S1. Work-flow in imaging quantification**

**Supplementary Figure S2. The calcium peak magnitude (A) and percentage of responding cells (B) during hypotonic swelling stimulation**

As shown above, vehicle treated MC3T3-E1 cells responded to hypotonic stress with a transit increased in [Ca²⁺]. While the 8br-cAMP treatment significantly increased both calcium peak magnitude of [Ca²⁺]ᵢ transients and the percentage of responding cells. Co-treating cells with PKI inhibited the effect of 8br-cAMP on calcium peak magnitude of [Ca²⁺]ᵢ transients and attenuated this effect on the percentage of responding cells.