Effects of Mechanical Strain on the Function of Gap Junctions in Osteocytes Are Mediated through the Prostaglandin EP2 Receptor*

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Osteocytes embedded in the matrix of bone are thought to be mechanosensory cells that translate mechanical strain into biochemical signals that regulate bone modeling and remodeling. We have shown previously that fluid flow shear stress dramatically induces prostaglandin release and COX-2 mRNA expression in osteocyte-like MLO-Y4 cells, and that prostaglandin E2 (PGE2) released by these cells functions in an autocrine manner to regulate gap junction function and connexin 43 (Cx43) expression. Here we show that fluid flow regulates gap junctions through the EP2 receptor EP2 activation of cAMP-dependent protein kinase A (PKA) signaling. The expression of the EP2 receptor, but not the subtypes EP1, EP3, and EP4, increased in response to fluid flow. Application of PGE2 or conditioned medium from fluid flow-treated cells to non-stressed MLO-Y4 cells increased expression of the EP2 receptor. The EP2 receptor antagonist, AH6809, suppressed the stimulatory effects of PGE2 and fluid flow-conditioned medium on the expression of the EP2 receptor, on Cx43 protein expression, and on gap junction-mediated intercellular coupling. In contrast, the EP3 receptor agonist butaprost, not the E1/E3 receptor agonist sulprostone, stimulated the expression of Cx43 and gap junction function. Fluid flow conditioned medium and PGE2 stimulated cAMP production and PKA activity suggesting that PGE2 released by mechanically stimulated cells is responsible for the activation of cAMP and PKA. The adenylate cyclase activators, forskolin and 8-bromo-cAMP, enhanced intercellular connectivity, the number of functional gap junctions, and Cx43 protein expression, whereas the PKA inhibitor, H89, inhibited the stimulatory effect of PGE2 on gap junctions. These studies suggest that the EP2 receptor mediates the effects of autocrine PGE2 on the osteocyte gap junction in response to fluid flow-induced shear stress. These data support the hypothesis that the EP2 receptor, cAMP, and PKA are critical components of the signaling cascade between mechanical strain and gap junction-mediated communication between osteocytes.

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Mechanical usage of bone results in strain-induced endogenous signals for bone modeling and remodeling. These signals are sensed by a proposed “mechanostat” in bone, of which the osteocyte is the cellular component (1–4). Gap junction-mediated intercellular communication has been proposed to play a role in determining the set point for the bone mechanostat (5). Osteocytes are dispersed throughout the mineralized matrix and connected to neighboring osteocytes via the extensive network of long slender cell processes. The cell processes of osteocytes are connected to each other and to the cells at the bone surface via gap junctions (6).

Gap junctions are transmembrane channels that connect the cytoplasm of two adjacent cells. These channels permit molecules with a molecular mass of less than 1 kDa, such as small metabolites, ions, and intracellular signaling molecules (i.e. calcium, cAMP, and inositol triphosphate), to pass through (7). These channels have been demonstrated to be important in modulating cell and tissue functions in many organs (8). Gap junction channels are formed by members of a family of proteins known as connexins (9, 10). The morphological proof of the existence of gap junction structures and the expression of connexins have been obtained for osteocytes (6, 11, 12). Cx43 has been reported to localize on the membranes of both the cell body and dendritic processes of osteocytes (13).

The application of force to bone results in several potential forms of mechanical stimuli for osteocytes including hydrostatic pressure, direct cell strain, and fluid flow-induced shear stress. Over the past few years, a number of theoretical and experimental studies support the hypothesis that flow of interstitial fluid is the major stimuli for osteocytes in response to loading (14–16). It has been found that mechanical forces applied to bone cause fluid to flow through the canaliculi surrounding the osteocyte and that fluid flow is probably responsible for deformation in the extracellular matrix and of the cell membrane (16, 17). Primary chicken osteocytes have been shown to be more sensitive than osteoblasts with respect to release of prostaglandins in response to either hydrostatic compression or to fluid flow shear stress, with fluid flow being more effective (18, 19). Osteocytes are deeply embedded in the mineralized bone matrix and are not readily accessible for many experimental approaches. An osteocyte-like cell line named MLO-Y4 has been established and characterized, which appears to have characteristics of primary osteocytes (20). More importantly, MLO-Y4 cells express an osteocyte-specific...
that fluid flow increases the release of PGE2 in MLO-Y4 cells osteocyte-like MLO-Y4 cells (23). Furthermore, we have shown intercellular communication and increases Cx43 expression in flow-induced shear stress stimulates gap junction-mediated function and Cx43 expression in osteoblast-like UMR106-01 MLO-Y4 cells. We have shown that MLO-Y4 cells are functionally coupled and that this coupling is mediated by gap junction channels (23). In addition, studies by Yellowley et al. (22) have reported that MLO-Y4 cells can couple through gap junctions to osteoblast-like MC3T3-E1 cells. Therefore, this cell line is a valuable model to study the function of gap junctions in cell-to-cell communication between osteocytes and other bone cells in response to mechanical strain.

Primary osteocytes and primary calvarial bone cells have been shown to release prostaglandins in response to fluid flow treatment (18, 19, 24). Prostaglandins are generally thought to be skeletal anabolic agents, because administration of these agents can increase bone mass in various animal species (25–27), stimulate bone formation in vitro in organ culture (28), and increase nodule formation in rat calvarial osteoblasts (29, 30). Prostaglandins also have catabolic effects on bone and have been shown to stimulate osteoclastic bone resorption and osteoclast formation and activation (28, 31). It has been shown to release prostaglandins in response to fluid flow and mediates the autocrine effects of PGE2 (28). Moreover, we identified intracellular cAMP and activated PKA as the signaling mediators between mechanical strain, autocrine PGE2, and gap junction-mediated communication in osteocytes.

**Experimental Procedures**

**Materials**—Tissue culture medium, HBSS, protein and RNA standards, Superscript II reverse transcriptase, and Taq DNA polymerase were purchased from Invitrogen. Fetal bovine serum and calf serum were from HyClone Laboratories (Logan, UT). BD (10 kDa) and LY (547 Da) were from Molecular Probes (Eugene, OR). TRI REAGENT was obtained from Molecular Research Center (Cincinnati, OH). QIAquick Gel Extraction kit was from Qiagen (Santa Clarita, CA). Paraformaldehyde (16% stock solution) was obtained from Electron Microscopy Science (Fort Washington, PA). Nitrocellulose membrane was from Schleicher & Schuell, and nylon transfer membrane-Hybond H+ was from Oncor (Gaithersburg, MD). Polyether sheets used for fluid flow assays were from Regal Plastics (San Antonio, TX). X-Omat AR films were from Eastman Kodak Co. Rat tail collagen type I, 0.15 mg/ml surfaces. The wall shear stress experienced by cells in these chambers was directly related to the flow rate of the circulating medium through the channel and inversely to the square of the channel height. Flow was gravity-driven, and the rate was governed by the height of separation between the upper and lower
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medium reservoirs using a peristaltic pump (Cole-Parmer Instrument, Chicago, IL) to return medium to the upper reservoir. Flow rate was continually monitored by an in-line flowmeter (Cole-Parmer). By using this flow system, wall shear stress levels caused by steady laminar flow of 16 dynes/cm² were generated by adjusting the channel height (using spacers) and medium flow rate (1–1.8 ml/s). The construction design of these chambers also permitted continuous microscopic visualization and recording of cells residing within the flow channel during shear stress regimens to monitor loss or change in cell orientation. All experiments were repeated at least three times. The circulating medium was identical to the culture medium. The entire flow system was housed within a large walk-in CO₂ incubator to maintain the circulating medium and the cell environment at 37 °C and pH 7.4. Upon completion of the flow regimen, the cell-covered sheets were removed for the analyses described below.

RT-PCR and Northern Blot Analyses—Total RNA was isolated from the cultures of MLO-Y4 cells using TRI REAGENT according to the manufacturer’s instructions. For RT-PCR analyses, cDNA was synthesized from 5 μg of the total RNA in a 20-μl reaction mixture containing 1× first strand buffer (20 μM Tris-HCl, 50 mM KCl, 25 mM MgCl₂, pH 8.4), 500 μM deoxy-NTPs, 10 μM dithiothreitol, 50 ng of oligo(dT)₁₄₋₁₈ primers, and 280 units of Superscript II reverse transcriptase. 0.2% cDNA was amplified by using PCR in a 50-μl reaction mixture containing 1× PCR buffer, 10 mM 5′ and 3′ primer, 200 μM deoxy-NTPs, 1 mM MgCl₂, and 2.5 units of Taq DNA polymerase. Amplifications were performed in a DNA Thermal Cycler (Techne) for 20, 25, 30, 35, and 40 cycles following the reaction profile of 94 °C for 30 s, 58 °C for 1 s, and 72 °C for 45 s. From preliminary experiments, we determined that the linear range of the PCR cycles versus products is between 20 and 30 cycles (data not shown). The PCR products presented in Figs. 1 and 3 are from 25 cycles of PCRs. The primers for EP₁, EP₂, EP₃, EP₄, and control β-actin genes were used exactly as described previously (42, 44). PCR-amplified fragments were size-selected and purified from 1% agarose gel using the QIAquick Gel Extraction kit according to the manufacturer’s instructions. Membranes were incubated with a 1:250 dilution of affinity-purified anti-Cx₄₃ antibody as described previously (46). Poly(A)⁺ RNAs were prepared using Micro Poly(A) Pure™ kit according to the manufacturer’s instructions. Thirty micrograms of total RNA or 3 μg of poly(A)⁺ RNA were loaded and separated by 1% agarose gel electrophoresis containing formaldehyde and transferred to a nylon membrane. The membrane was hybridized at 45 °C for 24 h in a hybridization solution containing 50% formamide and 32P-labeled cDNA probe corresponding to nucleotides 338—1037 of EP₂. The probed membrane was washed in a high stringency solution of 0.1% SDS and 0.1% SSC at 68 °C for 1 h.

SDS-PAGE and Western Blotting—The protein concentration of crude membrane samples of MLO-Y4 cells was determined using the Micro-BCA assay according to the manufacturer’s instructions. Equal amounts of protein (10 μg) were loaded in each lane of a 10% SDS-PAGE and transferred to nitrocellulose membranes according to the method of White et al. (46). Membranes were incubated with a 1:250 dilution of affinity-purified anti-Cx₄₃ antibody as described previously (47), a 1:250 dilution of anti-EP₂ receptor polyclonal antibody, or a 1:5,000 dilution of monoclonal anti-β-actin antibody. The primary antibody was detected using peroxidase-conjugated secondary anti-rabbit

![Fig. 2. Stimulation of prostaglandin receptor EP₂ mRNA expression by fluid flow in MLO-Y4 cells as shown by Northern blot analyses. MLO-Y4 cells were subjected to 2 h of steady fluid flow (FF) at 16 dynes/cm², and the cells were then harvested at 0 (lanes 1 and 2), 0.5 (lanes 3 and 4), 2 (lanes 5 and 6), and 24 h (lanes 7 and 8) after the fluid flow treatment. Total RNA was isolated from these cultures. Thirty micrograms of RNA were hybridized with a [α-32P]DNA probe of EP₂ receptor and β-actin under high stringency conditions. The size standards are indicated on the left. The intensity of each band on Northern blots was quantified by densitometry using NIH image software, and the ratio of band intensity of EP₂ receptor to β-actin is shown on the y axis.](image)

![Fig. 3. Stimulation of EP₂ receptor expression by fluid flow-conditioned medium as determined by RT-PCR and Western blots. Conditioned medium (CM) was collected from MLO-Y4 cells after 2-h of fluid flow (FF) shear stress (+) or unstressed control cells (−) and was incubated with unstimulated MLO-Y4 cells for 1 (lanes 1 and 2) and 24 h (lanes 3 and 4). A, total RNA was isolated from these cultures, and single-stranded cDNA was prepared. RT-PCR experiments were performed using specific DNA primers for EP₂ and β-actin. B, the cells were lysed and crude membranes prepared. Western blot analysis was performed using anti-EP₂ receptor or anti-β-actin antibody.](image)
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Fig. 4. Stimulation of EP<sub>2</sub> receptor expression by PGE<sub>2</sub> as determined by RT-PCR and Western blot analyses. Total RNA was isolated from cultures, and single-stranded cDNA was prepared. RT-PCR experiments were performed using specific DNA primers for EP<sub>2</sub> receptor (A and B), EP<sub>3</sub> receptor (B), and β-actin (A and B). A, MLO-Y4 cells treated with PGE<sub>2</sub> at the concentrations of 0 (lane 1), 0.1 (lane 2), 1 (lane 3), 2.5 (lane 4), 5 (lane 5), and 10 μM (lane 6) for 4 h. B, MLO-Y4 cells treated in the absence (−) or presence (+) of 5 μM PGE<sub>2</sub> for (lanes 1 and 2), 4 (lanes 3 and 4), and 24 h (lanes 5 and 6). The cells were also lysed and crude membranes prepared. Western blot analysis was performed using anti-EP<sub>2</sub> or anti-β-actin antibody. C, MLO-Y4 cells were treated with PGE<sub>2</sub> at concentrations of 0 (lane 1), 0.01 (lane 2), 0.1 (lane 3), 1 (lane 4), 2.5 (lane 5), 5 (lane 6), and 10 μM (lane 7).

or anti-mouse antiserum followed by use of a chemiluminescence reagent kit (ECL) according to the manufacturer’s instructions. The membranes were exposed to X-Omat AR films and detected by fluorography. The band intensity was quantified by densitometry (NIH image).

Scrape-loading Dye Transfer Assay and Fluorescence Microscopy—MLO-Y4 cells were grown to ~75–85% confluence. The scrape-loading dye transfer assay was performed based on the modified procedure described by El-Fouly et al. (49). In this method, cells were scratched in the presence of two types of fluorescence dyes: Lucifer Yellow (LY) (457 Da), which can penetrate through gap junction channels, and rhodamine dextran (RD) (10 kDa), which is too large to pass through the channels, thus serving as a tracer dye for the cells originally receiving the dye. Cells were washed three times with HBSS plus 1% bovine serum albumin. Then 1% LY and 1% RD dissolved in PBS were applied to the cells, which subsequently were scraped lightly with a 26-gauge needle. After incubation for 10 min, cells were washed with HBSS three times, then with PBS twice, and finally fixed in fresh 2% paraformaldehyde (from 16% stock) for 20 min. The dye transfer results were examined using a fluorescence microscope (Zeiss Axioscope, Zeiss, Jena, Germany), in which LY could be detected by using the filter set for fluorescein and RD using the filter set for rhodamine. To reach statistical significance, more than 500 cells receiving RD were counted for each assay.

cAMP Immunoassay and Analysis of PKA Activity—MLO-Y4 cells were grown to a density where only the dendritic processes and not the cell body made contact in 6-well 35-mm culture plates. Cells were treated with 0.2 mM isobutylmethylxanthine, 0.2 mM ethanol (vehicle), PGE<sub>2</sub>, forskolin, or fluid flow-conditioned medium for various times at 37 °C. The cells were washed with cold PBS and then harvested in ice-cold ethanol. The collected samples were vacuum-dried and resuspended in assay buffer as suggested in the kit. The amount of the intracellular cAMP was determined using cAMP EIA kit according to the manufacturer’s instructions.

The activity of protein kinase A was determined as described previously (48). Briefly, MLO-Y4 cells were washed with cold PBS, lysed in HP buffer (10 mM potassium phosphate, pH 6.8, 1 mM β-mercaptoethanol, 10 μg/ml leupeptin, 10 mM magnesium acetate, 10 μM ATP, and 300 μg/ml Kemptide substrate), and then homogenized by passing 15 times through a 26-gauge needle. The homogenates were centrifuged at 8,000 rpm at 4 °C for 5 min, and the supernatant was collected for PKA assay. The activity of PKA was measured in a total volume of 100 μl containing 5 × 10<sup>5</sup> cpm [γ-<sup>32</sup>P]ATP (3,000 Ci/mmol). The reaction mixtures were incubated at 30 °C for 5 min, and 30 μl of reaction mixture was spotted onto a Whatman P-81 paper. The paper was washed in 75 mM phosphoric acid twice, acetone once, and then subjected to scintillation counting for measurement of the levels of Kemptide phosphorylation.

Statistical Analysis—Data were analyzed using one-way analysis of variance and Bonferroni comparison test with the Instat biostatistic program (GraphPad software). Data are presented as the mean ± S.D. of three determinations. In the figures, asterisks indicate the degree of significant differences compared with the controls (*, p < 0.05, **, p < 0.01, and ***, p < 0.001).
RESULTS

Fluid Flow-induced Shear Stress Stimulates the Expression of Prostaglandin Receptor Subtype EP2, but Not Other Subtypes in Osteocyte-like MLO-Y4 Cells—In our previous studies, PGE2 released by mechanically stressed MLO-Y4 cells functioned in an autocrine manner to enhance the activity of gap junctions and increase the expression of Cx43 protein (42). To determine the PGE2 receptor subtype responsible for this autocrine effect, the expression of four known subtypes of PGE2 receptors (EP1, EP2, EP3, and EP4) (36, 37) was analyzed. RT-PCR was performed with RNA isolated from MLO-Y4 cells at various post-stress time points after these cells were treated with fluid flow—induced shear stress (Fig. 1, lanes 1 and 2) or presence (lanes 3 and 4) of PGE2 and/or 5 μM AH6809 (lanes 2 and 4). The EP2 receptor agonist, butaprost, but not the EP1/EP3 receptor agonist, sulprostone, stimulated Cx43 expression as determined by Western blot analyses.

Fluid Flow-conditioned Medium and PGE2 Stimulate the Expression of EP2, but Have No Effect on Other Subtypes of Prostaglandin Receptor—We have reported previously (42) that PGE2 released by fluid flow-stimulated MLO-Y4 cells functioned in an autocrine manner on gap junctions. To determine whether the stimulatory effect of fluid flow on EP2 expression is due to the release of PGE2, we first determined the expression levels of EP2 in the presence and absence of conditioned medium generated from fluid flow-treated cells (Fig. 3) (n = 3). Compared with β-actin levels (Fig. 3A, β-actin, lanes 1–4), an increase in EP2 mRNA expression was observed 1 h after the fluid flow-conditioned medium treatment (Fig. 3A, EP2, lanes 1 and 2) and became more apparent after 24 h (Fig. 3A, EP3, EP4, lanes 3 and 4). Western blot analysis also showed a similar increase in EP2 protein expression 24 h after treatment with fluid flow-conditioned medium (Fig. 3B, lanes 3 and 4), whereas no effect was observed after 1 h of treatment although there was an increase in mRNA (Fig. 3B, lanes 1 and 2). Treatment with PGE2 up-regulated EP2 mRNA and protein expression in a dose- and time-dependent manner (Fig. 4) (n =

![Image](image-url)
3). Various concentrations of PGE$_2$ were applied to MLO-Y4 cells for 4 h, and the expression of EP$_2$ mRNA was determined (Fig. 4A). EP$_2$ expression was increased significantly with increasing concentrations of PGE$_2$. The concentration that gave the maximal increase is around 5–10 μM, which is similar to the PGE$_2$ concentration required for the up-regulation of gap junctions that we have observed in previous studies (42). There were no changes in expression of β-actin (Fig. 4A, β-actin). The increase of EP$_2$ mRNA was observed 1 h after treatment with 5 μM PGE$_2$ (Fig. 4B, EP$_2$, lanes 1 and 2), and this increase was more evident 4 and 24 h after treatment (Fig. 4B, EP$_2$, lanes 3–6). In contrast, the expression of EP$_3$ and β-actin was unchanged during the same periods (Fig. 4B, EP$_3$ and β-actin,

3). The EP$_2$ receptor antagonist, AH6809, blocked the stimulatory effect of PGE$_2$ and fluid flow-conditioned medium on gap junction intercellular coupling. Scrape-loading dye transfer experiments were performed to analyze intercellular coupling. A, MLO-Y4 cells were treated in the absence (Ctrl) or presence of PGE$_2$ and/or 5 μM AH6809. B, MLO-Y4 cells were treated in the absence (Ctrl) or presence of fluid flow-conditioned medium (CM/FF) and/or 5 μM AH6809. C, MLO-Y4 cells were treated in the absence (Ctrl) or presence of 5 μM butaprost or 5 μM sulprostone. The relative degree of dye transfer as compared with control untreated cells is presented. **, p < 0.01. All data are presented as mean ± S.D. and n = 3.

Fig. 6. The EP$_2$ receptor antagonist, AH6809, blocked the stimulatory effect of PGE$_2$ and fluid flow-conditioned medium on gap junction intercellular coupling. Scrape-loading dye transfer experiments were performed to analyze intercellular coupling. A, MLO-Y4 cells were treated in the absence (Ctrl) or presence of PGE$_2$ and/or 5 μM AH6809. B, MLO-Y4 cells were treated in the absence (Ctrl) or presence of fluid flow-conditioned medium (CM/FF) and/or 5 μM AH6809. C, MLO-Y4 cells were treated in the absence (Ctrl) or presence of 5 μM butaprost or 5 μM sulprostone. The relative degree of dye transfer as compared with control untreated cells is presented. **, p < 0.01. All data are presented as mean ± S.D. and n = 3.

Fig. 7. PGE$_2$ and fluid flow-conditioned medium elevate intracellular cAMP levels in MLO-Y4 cells. Intracellular levels of cAMP were measured using cAMP EIA kit. A, MLO-Y4 cells were treated in the absence (Ctrl) or presence of 5 μM PGE$_2$ for 15, 30, and 45 min. A time-dependent increase in intracellular cAMP was observed. B, MLO-Y4 cells were treated with PGE$_2$ at the concentration of 0, 0.5, 1, 5, 10, and 20 μM for 30 min. A concentration-dependent increase in cAMP was observed. C, MLO-Y4 cells were treated in the absence (Ctrl) or presence of fluid flow-conditioned medium (CM/FF) for 0.5 or 1 h. A significant increase in intracellular cAMP was observed in response to fluid flow-conditioned media at both time points. *, p < 0.05; **, p < 0.01). All data are presented as mean ± S.D. and n = 3.
centrations of AH6809 suppressed the stimulatory effect of medium was completely blocked by AH6809 (Fig. 6). Stimulation of intercellular coupling by fluid flow-conditioned protein levels of EP2 were also increased with increasing concentrations of PGE2. A lower concentration of PGE2, 0.01 μM, stimulated an increase in the expression of EP2 protein even though this concentration had no effect on EP2 mRNA expression (Fig. 4C, lanes 1 and 2). This increase is less dramatic than when higher concentrations of PGE2 were used (Fig. 4C, lanes 3–7). The upper migrating band shown in Fig. 4C could be a contaminating protein because a similar nonspecific protein band is also seen in the product information sheet provided by Cayman Chemicals.

This observation indicates that the regulation of the EP2 receptor expression by PGE2 not only occurs at the mRNA level but also at the protein level. These results suggest that PGE2 released by fluid flow-treated MLO-Y4 cells is responsible for the increased gene and protein expression of prostaglandin receptor subtype EP2.

**Blocking EP2 Receptor Activation Suppresses the Stimulatory Effect of PGE2 on Cx43 Expression and Gap Junction Function**—To determine whether up-regulation of Cx43 expression and gap junction function by PGE2 released in response to fluid flow shear stress is mediated through EP2 receptor, we took advantage of antagonists and agonists specific for EP receptor-mediated signaling. AH6809, a well characterized antagonist for EP1 and EP2-mediated signaling (54, 55), was used to treat MLO-Y4 cells. As shown in Fig. 4, PGE2 increased EP2 receptor mRNA (Fig. 5A, EP2, lanes 1 and 2), whereas the expression of β-actin was not altered (Fig. 5A, β-actin). This up-regulation of EP2 receptor expression by PGE2 was decreased with increasing concentrations of AH6809 (1 and 5 μM) (Fig. 5A, lanes 3 and 4), whereas β-actin expression was not affected. Compared with the expression of β-actin, the stimulatory effect of PGE2 on Cx43 protein expression was also decreased by treatment with AH6809 (Fig. 5B). The EP2 receptor antagonist SC-19220 (56) did not affect the expression of Cx43 (data not shown), showing that the EP2 receptor is responsible for the observed effects. To validate further that these effects of fluid flow shear stress are mediated through the EP2 receptor, cells were pre-treated with the EP2 antagonist, AH6809, prior to treatment with fluid flow-conditioned medium. Consistently, fluid flow-conditioned medium stimulated Cx43 expression compared with β-actin controls (Fig. 5C, lanes 1 and 2). Increased concentrations of AH6809 suppressed the stimulatory effect of fluid flow-conditioned medium on Cx43 expression (Fig. 5C, lanes 3–6), suggesting that up-regulation of Cx43 by fluid flow is mediated through the activation of EP2 receptor-mediated signaling. Two agonists, butaprost, specific for EP2 signaling, and sulprostone, specific for EP2/EP3 signaling, were also used to further confirm the exclusive activation of EP2 receptor signaling in osteocytes. Compared with β-actin controls, increasing concentrations of the EP2 agonist, butaprost, stimulated the expression of Cx43, but no effect was observed with the EP2/EP3 agonist, sulprostone (Fig. 5D).

The function of gap junctions was determined by scrape-loading dye transfer analysis. The PGE2-induced up-regulation of gap junction-mediated intercellular coupling was attenuated by EP2 receptor antagonist AH6809 (Fig. 6A). Consistently, the stimulation of intercellular coupling by fluid flow-conditioned medium was completely blocked by AH6809 (Fig. 6B). The EP2 receptor agonist, butaprost, stimulated gap junction function, whereas the EP1/EP3 receptor agonist, sulprostone, had no effect on gap junctions (Fig. 6C). Together, these results support our hypothesis that the activation of EP2 receptor is essential for the up-regulation of gap junction function and Cx43 expression in response to fluid flow shear stress.

**PGE2 and Conditioned Medium from Fluid Flow-treated MLO-Y4 Cells Enhance Intracellular cAMP Production**—The activation of PGE2 receptor subtype EP2 leads to an increase in intracellular cAMP (36, 40). To determine whether the action of PGE2 in osteocytes leads to an increase in intracellular cAMP, MLO-Y4 cells were treated with PGE2 for 15, 30, and 45 min.

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**Fig. 8. Stimulation of PKA activity in MLO-Y4 cells by the adenylate cyclase analogs, 8-Br-cAMP and forskolin, by PGE2, and by fluid flow-conditioned medium.** PKA activity was measured, and the relative percent change as compared with non-treated control is shown on the y axis. A, the cAMP analog, 8Br-cAMP, was applied to MLO-Y4 cells for 30 min in the absence or presence of the specific PKA inhibitor, H89 (2 μM). The controls (Ctrl) were treated similarly without the addition of 8Br-cAMP. B, MLO-Y4 cells were treated in the absence (Ctrl) or presence of 5 μM PGE2 or 10 μM forskolin (FSK) for 30 min. C, MLO-Y4 cells were treated with (+) or without (−) fluid flow-conditioned medium for 30 min. PKA activity was significantly increased by the adenylate cyclase analogs, PGE2, fluid flow-conditioned medium. *, p < 0.05; **, p < 0.01. All data presented as mean ± S.D. and n = 3.
Increased production of intracellular cAMP was observed at all time points (Fig. 7A). The most significant increase in cAMP production occurred 30 min after PGE2 application. The induction of cAMP was also examined in MLO-Y4 cells treated with various concentrations (0.5–20 μM) of PGE2 (Fig. 7B). The amount of cAMP induction was generally directly proportional to the amount of PGE2 applied, with optimal concentrations of 5–20 μM, a concentration range that we have previously observed to be necessary for maximal stimulation of gap junction function (42) and now observe for EP2 receptor expression (Fig. 4, A and C). A similar increase in the levels of intracellular cAMP was observed with the 30 min and 1 h of treatment with fluid flow-conditioned medium (Fig. 7C).

Adenylate Cyclase Activators, PGE2, and Fluid Flow-conditioned Medium Increase Intracellular PKA Activity in MLO-Y4 Cells—Intracellular CAMP has been recognized as one of the major regulators for PKA; however, recent studies (50, 51) also suggest other roles for cAMP, which may not be necessarily related to PKA activation. A) Compared with non-treated control cells, a dramatic increase in PKA activity was observed in cells treated with 8-Br-cAMP. This increase was blocked by a PKA-specific inhibitor, H89, which confirms the specificity of this stimulatory effect of cAMP on PKA activity. PKA activity was also increased in a similar manner by another adenylate cyclase activator, forskolin (data not shown). Together, these results suggest that cAMP is the activator for PKA in MLO-Y4 cells.

To verify that autocrine PGE2 released by fluid flow-stimulated MLO-Y4 cells can directly activate PKA, MLO-Y4 cells were treated with PGE2, and the activity of PKA was determined (Fig. 8B). PKA activity was significantly increased in cells treated with PGE2 and with the adenylate cyclase activator, forskolin. Like PGE2, fluid flow-conditioned medium also enhanced the kinase activity of PKA (Fig. 8C). This observation supports the hypothesis that the stimulatory effect by autocrine PGE2 released by fluid flow-stimulated cells is likely to be mediated by a cAMP-coupled PKA pathway.

Activators of Adenylate Cyclase Increased Cx43 Protein Expression, and the Stimulatory Effect of PGE2 on Cx43 Expression Was Blocked by the PKA Inhibitor H89—Our previous studies (42) have shown that PGE2 and fluid flow-conditioned medium increase Cx43 expression and functional gap junctions. If cAMP-activated PKA is the downstream effector of PGE2, then the activation of this signaling pathway would stimulate Cx43 expression and gap junction function in a similar fashion as PGE2. The expression of Cx43 protein was enhanced upon treatment of MLO-Y4 cells with adenylate cyclase activators (Fig. 9, A–D). Western blot analysis of forskolin-treated MLO-Y4 cells showed a time- and dose-dependent (Fig. 9B, Cx43) increase in Cx43 expression. The level of the control protein, β-actin, remained relatively constant. A similar time- and dose-dependent increase in Cx43 expression was also observed with another adenylate cyclase activator, 8 Br-cAMP (Fig. 9, C and D). To determine whether increased PKA activity is responsible for the increase in Cx43 expression induced by PGE2, MLO-Y4 cells were treated with an inhibitor of PKA, H89. A significant inhibitory effect by H89 on up-regulation of Cx43 by PGE2 was detected (Fig. 9E). These results show that PGE2-induced activation of the downstream mediators of EP2 receptor signaling, cAMP and PKA, is responsible for the increase in the expression of Cx43.

Activators of Adenylate Cyclase Enhance Interacellular Connections and Stimulate Gap Junction Function—Compared with control cells (Fig. 10A, panel a), alterations in cell mor-
phology were observed in MLO-Y4 cells treated with the adenylate cyclase activator, forskolin (Fig. 10A, panel b). Treatment of the MLO-Y4 cells with forskolin elongated the dendritic processes and enhanced intercellular connectivity between neighboring cells (open arrowheads). These morphological changes are similar to MLO-Y4 cells treated with fluid flow-conditioned medium and PGE2 (Fig. 10A, panels c and d) or cells subjected to fluid flow (42). The increased connectivity in the forskolin-treated MLO-Y4 cells correlates with increased intercellular coupling mediated by gap junctions as determined by scrape-loading dye transfer analysis (Fig. 10B). This increase was significantly blocked by the PKA-specific inhibitor H89, suggesting that the observed increase in gap junction function is mediated through a cAMP-PKA signaling pathway. Together, the results of these biochemical analyses, showing an increase in Cx43 protein expression and an increase in gap junction function, support the hypothesis that PGE2 mediates its effects through the induction of cAMP followed by PKA activation.

**DISCUSSION**

We have shown previously that PGE2 released by mechanically stimulated MLO-Y4 cells functions in an autocrine fashion to up-regulate gap junction function and Cx43 expression (42). Here we demonstrate that autocrine PGE2 exerts its effects on gap junctions through the EP2 receptor-coupled cAMP-PKA pathway. There are three major novel findings in this study. 1) Fluid flow shear stress, fluid flow-conditioned medium, and PGE2 increase the expression of EP2 receptor and not the expression of any other EP receptor subtypes. 2) The effects of fluid flow shear stress, fluid flow-conditioned medium, and PGE2 on osteocyte gap junctions are mediated through activation of EP2 receptors. 3) The effect of EP2 receptor activation on gap junctions is mediated through downstream cAMP-PKA signaling. Based on our previous studies (23, 42) and current observations, a model is proposed for the functional involvement of an EP2 receptor-activated signaling mechanism in the regulation of gap junctions in response to mechanical stimulation of osteocytes, as illustrated in Fig. 11. Application of mechanical strain to osteocytes results in the redistribution of Cx43, increased assembly of gap junction channels, and increased formation of additional gap junctions (23). Within minutes, detectable PGE2, converted from arachidonic acid, is released from the cell. The released PGE2 functions predominantly in an autocrine fashion on osteocytic gap junctions. We have shown previously that at 2 h of fluid flow shear stress, gap junction-mediated intercellular communication is increased when there is no detectable changes in Cx43 expression (23). However, a significant increase in Cx43 pro-
tein does occur at 24 h after fluid flow treatment. Increased biosynthesis of Cx43, in turn, generates additional functional gap junctions, which can accommodate the passage of greater numbers of signaling molecules between osteocytes. In addition, transcription of inducible prostaglandin synthase, COX-2, is also increased during the post-stress period, through the autocrine effects of newly formed PGE$_2$ (42). The studies presented here bridge the gap between the predominant effect of autocrine PGE$_2$ released in response to mechanical strain and the stimulation of gap junctions. Here we show that autocrine PGE$_2$ released in response to mechanical stimulation activates the EP$_2$ receptor, which in turn activates the cAMP-coupled signaling cascade and, in turn, the activation of PKA. This pathway combined with other minor uncharacterized pathway(s) ultimately leads to the increased expression of Cx43 and the formation of a greater number of functional gap junction channels.

We observed that the treatment of MLO-Y4 cells with PGE$_2$ increased intracellular levels of cAMP. It is known that, similar to the PGE$_2$ receptor subtype EP$_2$, activation of EP$_4$ also leads to an increase of intracellular cAMP (39). Thus far, there is no commercially available antagonist specific only for EP$_2$. Therefore, we took advantage of other receptor antagonists: AH6809 that is specific for both EP$_1$ and EP$_2$ and SC-19220 that is specific for only EP$_1$. These reagents have been reported to block EP$_1$ and EP$_2$ signaling but not EP$_4$ signaling in cells and in whole animals (54, 57). Moreover, unlike EP$_2$, activation of the human EP$_1$ receptor leads to signaling through inositol 1,4,5-trisphosphate generation and increased Ca$^{2+}$ but not through increased intracellular cAMP (58). To confirm further the specific functional involvement of the EP$_2$ receptor, we used the recently released EP$_2$-specific agonist butaprost and the EP$_4$/EP$_3$ agonist sulprostone. The stimulatory effect on Cx43 expression was only observed with the treatment of the EP$_2$ agonist but not with the EP$_4$/EP$_3$ agonist. Therefore, these antagonist and agonist reagents can be effectively used to distinguish the distinct functions and signaling pathways mediated by specific EPs. Together, these results suggest that fluid flow shear stress increases gap junction function via the EP$_2$ receptor.

We reported previously (42) that the concentration of PGE$_2$ released in the fluid-conditioned medium is ~10 nM after 2 h of shear stress. In this report, we show that exogenous PGE$_2$ at a similar concentration to that in the fluid-conditioned medium was also able to increase EP$_2$ receptor expression. However, the magnitude of the stimulation of EP$_2$ receptor expression by fluid flow-conditioned medium is higher than the stimulation by exogenous PGE$_2$ at a similar concentration. This suggests that another factor, in addition to prostaglandins, is likely to play an additive or synergistic role in stimulation of EP$_2$ receptor expression in response to mechanical strain. These observations are supported by our previously published work (42) that PGE$_2$-depleted fluid flow-conditioned medium had a reduced effect on gap junctions and that indomethacin, an inhibitor of prostaglandin synthesis, significantly but not completely blocked the stimulatory effect of fluid flow-conditioned medium on gap junctions. An antagonist of the EP$_2$ receptor completely abolished the effect of PGE$_2$ and fluid flow-conditioned medium on Cx43 expression and gap junctions, suggesting that PGE$_2$ along with other unidentified factor(s) predominantly function through the EP$_2$ receptor to regulate gap junctions. These data support the hypothesis that EP$_2$ receptor signaling activated mainly by PGE$_2$ released in response to fluid flow shear stress plays an essential role in the regulation of gap junctions.

In our study, the activity of gap junctions and the increase in Cx43 were mediated through the EP$_2$-receptor-activated cAMP-PKA pathway. We found that treatment with a PKA inhibitor only partially inhibited intercellular coupling and Cx43 expression. This observation could indicate that elevated intracellular cAMP may activate other pathways in addition to the PKA pathway. We observed that lower concentrations of FSK promoted intercellular coupling and Cx43 expression, whereas higher concentrations of FSK down-regulated gap junctions. At higher concentrations of FSK, the cells within our test period appear healthy with normal expression of proteins such as β-actin and Cx43. In support of our observations, cell toxicity caused by FSK in the concentration that we used has not been reported previously. The biphasic effect of FSK on protein expression and other cellular responses has been reported previously, such as the expression of testin in Sertoli cells, intracellular Ca$^{2+}$ in myocytes, and platelet aggregation (59–61).
The possible explanation for this phenomenon is that higher levels of cAMP induced by high concentrations of FSK lead to the production of mRNA-destabilizing proteins (62–64), which, in turn, leads to reduced overall protein expression. The cAMP-coupled PKA signaling pathway is frequently associated with increased gap junctional permeability and total number of gap junctions (65–67). In some types of cells, this increase in permeability correlates with an increase in the expression of Cx43 (68, 69). However, PKA activated by intracellular cAMP, may also regulate gap junctions at the level of channel assembly by increasing the numbers of junctional plaques on the cell surface (67, 70). A recent study (71) shows that increased levels of intracellular cAMP enhance gap junction assembly and junctional permeability in osteoblast-like cells. In the present studies, the cAMP-activated PKA may not directly phosphorylate Cx43 because the levels of Cx43 phosphorylation in terms of changes in the cAMP-activated PKA may not directly phosphorylate Cx43 (68, 69).

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29. Bastien, L., Sawyer, N., Grynspan, R., Metters, K., and Adam, M. (1994) Biochim. Biophys. Acta 122, 64, which, ... the presence of a negative charge at specific sites of Cx43 are a prerequisite for enhancement of gap junction formation by cAMP (72). Future studies will be directed toward exploring the regulatory mechanisms between EP2 receptor-activated cAMP-coupled PKA signaling and gap junction-mediated intercellular communication between osteocytes.