J Membrane Biol (2012) 245:401–409
DOI 10.1007/s00232-012-9462-2

Connexin43 and Pannexin1 Channels in Osteoblasts: Who Is the "Hemichannel"?

Mia M. Thi · Shalena Islam · Sylvia O. Suadicani · David C. Spray

Received: 9 April 2012 / Accepted: 20 June 2012 / Published online: 15 July 2012

Abstract Osteoblasts sense and respond to mechanical stimuli in a process involving influx and release of large ions and signaling molecules. Unapposed gap junction hemichannels formed of connexin43 (Cx43) have been proposed as a major route for such exchange, in particular for release of ATP and prostaglandin E_2 (PGE_2) in osteocytes. However, we have found that Cx43-null osteoblasts have unaltered, mechanically induced PGE_2 release and ATP-induced YoPro dye uptake. In contrast, PGE_2 release in response to fluid shear stress is abolished in $P2X_7$ receptor ($P2X_7R$)–null osteoblasts, and ATP-induced dye uptake is attenuated following treatment of wild-type cells with a $P2X_7R$ or Pannexin1 (Panx1) channel blocker. These data indicate that Panx1 channels, in concert with $P2X_7R$, likely form a molecular complex that performs the hemichannel function in osteoblast mechanosignaling.

Keywords $P2X_7R$ · ATP · Osteoblast · Gap junction · Dye uptake

M. M. Thi (✉)
Department of Orthopedic Surgery, Albert Einstein College of Medicine, Bronx, NY 10461, USA
e-mail: mia.thi@einstein.yu.edu

M. M. Thi · S. O. Suadicani · D. C. Spray
Department of Neuroscience, Albert Einstein College of Medicine, Bronx, NY 10461, USA

S. Islam
Department of Biology, The City College of New York, New York, NY 10031, USA

S. O. Suadicani
Department of Urology, Albert Einstein College of Medicine, Bronx, NY 10461, USA

Introduction

Gap junctions are formed in vertebrates by connexins and in invertebrates by innexins; remarkably, these functionally equivalent proteins display no homology at the level of amino acid sequence; but, as shown 40 years ago by Ross Johnson, their intramembrane particles look pretty similar (Johnson and Sheridan 1971; Johnson et al. 1973). Pannexins were discovered in searches of the vertebrate genome for similarities to innexin cDNA sequences. In contrast to connexins and innexins, pannexins likely do not form junctional channels; however, at least one of the three pannexins, Pannexin1 (Panx1), forms large-conductance, mechanosensitive and highly permeable channels in non-junctional membranes of mammalian cells (for reviews, see Iglesias et al. 2009a; Sosinsky et al. 2011). Certain connexin hemichannels can also open when unpaired, forming pores permeable to large molecules, similar to Panx1 channels and gap junctions (Spray et al. 2006).

Thus, whereas both connexins and Panx1 are involved in intercellular communication, they appear to have different roles (Scemes et al. 2007). Connexins mainly provide junctional coupling, whereas Panx1 channels assist autocrine/paracrine signaling by providing a pathway for controlled release of signaling molecules such as ATP (Dahl and Locovei 2006; Scemes et al. 2007; MacVicar and Thompson 2010; Sosinsky et al. 2011). In this article we report largely unpublished studies of osteoblasts in vitro, focusing on these different roles of connexins and Panx1 in bone cells. We conclude that, under the conditions of our studies, functions previously attributed to connexin43 (Cx43) hemichannels are likely mediated by Panx1 channels instead.

Bone cells are coupled into a functional syncytium by gap junction channels formed mainly by Cx43 (Donahue

2000; Civitelli 2008). Intercellular signals transmitted through gap junction channels formed by these connexins are believed to play key roles in bone embryogenesis, differentiation and mineralization (Minkoff et al. 1994; Donahue 2000; Schiller et al. 2001; Civitelli 2008; Kar et al. 2012). Studies of Cx43-deficient mice (Lecanda et al. 2000; Civitelli 2008) and our recent study with immortalized wild-type and Cx43-null osteoblasts (Thi et al. 2010b) have clearly demonstrated that the presence of Cx43 is essential during early phases of osteoblast differentiation and maturation.

Signaling through gap junction channels is also believed to be essential in bone remodeling. This life-long process is crucial for maintenance of bone mass and integrity and consists of continuous bone resorption and deposition, whereby aging tissue is replaced and injuries are repaired. While it is well established that bone remodeling is regulated by the mechanical loading imposed on the bone by daily physical activity, it is still unclear how these load-generated mechanical signals are translated into the cellular and biochemical events that ultimately result in bone remodeling. There is accumulating evidence that non-junctional Cx43 could actively participate in these events, where Cx43 hemichannels would open in response to mechanical stimulation and provide an efflux pathway for mechanosignaling molecules, such as ATP and prostaglandin E_2 (PGE_2) (Romanello and D'Andrea 2001; Jiang and Cherian 2003; Cherian et al. 2005; Genetos et al. 2007). Cx43 hemichannels are not mechanosensitive, and their response to mechanical stimuli has been proposed to be mediated by their interaction with integrins (Batra et al. 2012a). Besides Cx43 hemichannels, a role for ATP receptors (ionotropic $P2X_7$ receptors) in bone cell mechanotransduction and signaling has also been proposed (Li et al. 2005). Activation of $P2X_7$ receptors ($P2X_7Rs$) has been shown to mediate ATP-induced ATP release from certain cell types (Anderson et al. 2004; Suadicani et al. 2006), and $P2X_7R$ deletion abrogates PGE_2 release from osteoblasts in response to fluid shear stress (Li et al. 2005). Moreover, our studies and those of others have shown that $P2X_7Rs$ functionally interact with Panx1 channels to provide the permeabilization pathway for $P2X_7R$-induced ATP and IL-1β release (Pelegrin and Surprenant 2006; Locovei et al. 2007). In addition, Panx1 channels possess both mechanosensitivity (Bao et al. 2004) and activation by extracellular K^+ (Silverman et al. 2009; Suadicani et al. 2012), both of which may be important in bone pathophysiology.

The main goal of the studies described here was to determine the relative role of Cx43 and Panx1 in bone cell mechanotransduction and formation of the ATP-induced dye-uptake pathway that facilitates PGE_2 release. Use of pharmacological approaches to discriminate the participation of connexin hemichannels and Panx1 channels is somewhat complicated by the overlapping effects of pharmacological blockers. In this study we combined use of these drugs with that of the newly generated MOB cell line (wild-type and Cx43-null) (Thi et al. 2010b), allowing us to specifically address the participation of Cx43 in fluid shear stress–induced PGE_2 release from osteoblasts and ATP-induced dye uptake.

Materials and Methods

Materials

Alpha-minimal essential medium (α-MEM), fetal bovine serum (FBS), penicillin–streptomycin and YoPro-1 iodide (491/509) were purchased from Invitrogen (Carlsbad, CA). Collagenase type II was purchased from Worthington Biochemical (Lakewood, NJ), protease inhibitor cocktail was purchased from Roche (Mannheim, Germany), nitrocellulose membranes were purchased from Whatman (Dassel, Germany) and the Immobilon Western detection kit was purchased from Millipore (Billerica, MA). All other chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Cell Line Culture

Osteoblastic MOB-C and 43KO-MOB-C cells (mouse osteoblast cell lines derived from wild-type and Cx43-null calvaria) (Thi et al. 2010b) and MC3T3-E1 cells (subclone 4) (ATCC, Manassas, VA) were cultured in α-MEM containing 1 % penicillin–streptomycin and 10 % FBS at 37 °C with 95 % air/5 % CO_2.

Primary Osteoblast Culture

As described in our previous work (Thi et al. 2010b), osteoblasts were isolated from calvaria of newborn (P0) and embryonic (E19–20) wild-type and Cx43-null mice obtained from in-house mating of Cx43 heterozygous mice (C57BL/6J-Gja1^{tm1Kdr}) (Reaume et al. 1995) and from newborn $P2X_7R$-null mice (B6.129P2-P2rx7^{tm1Gab}/J). All animal procedures and experimental protocols were approved by the Institute for Animal Studies of the Albert Einstein College of Medicine in accordance with NIH guidelines. Briefly, pups were killed by decapitation, and the periosteum and endosteum of individual calvaria were carefully removed, cleaned and thoroughly diced into small pieces, then pooled for each pup and digested in 1× PBS containing 4 mg/ml of collagenase type II at 37 °C for 10 min. Supernatant from the second and third sequential

digestions at 37 °C were collected. Cells were then collected by centrifugation, resuspended in α-MEM supplemented with 10 % FBS and 1 % penicillin–streptomycin and seeded in culture dishes. Primary osteoblasts isolated from wild-type and from Cx43-null and $P2X_7R$-null calvarial tissue were termed PMOB, 43KO-PMOB and $P2X_7$RKO-PMOB, respectively.

Pulsatile Fluid Shear Stress Treatment

For flow experiments the immortalized wild-type (MOB-C), Cx43-null (43KO-MOB-C) and MC3T3-E1 osteoblastic cell lines were seeded at 10^4 cells/cm^2 and primary osteoblasts were seeded at 2×10^4 cells/cm^2 and grown on glass slides for 3 days. The fluid flow setup consisted of a parallel plate flow chamber (Cytodyne, La Jolla, CA) and a recirculating flow circuit as previously described (Thi et al. 2010a). Briefly, the flow loop included a variable speed Masterflex pump (Cole-Palmer Instrument, Vernon Hills, IL) and a reservoir with culture medium (α-MEM + 1 % FBS) maintained at 37 °C with 95 % air/5 % CO_2. This system produces pulsatile flow over a cell monolayer with average shear stress of 10 dyne/cm^2 at 1 Hz frequency. Control cells were kept under static conditions at 37 °C with 95 % air/5 % CO_2.

Quantification of PGE_2 Release

Supernatants from control (static) and pulsatile flow–conditioned medium were collected immediately after 1-h exposure of cells to fluid shear stress. Supernatants were stored at –80 °C and then assayed for PGE_2 using a PGE_2 EIA Kit (Cayman, Ann Arbor, MI). Average OD values were acquired at wavelength 415 nm using a FLUOStar Omega plate reader (BMG Labtech, Ortenberg, Germany). PGE_2 concentrations in the medium were determined from the standard curve obtained for each set of experiments. The amount of PGE_2 release was normalized to respective cellular protein levels. Total protein concentration from the samples was determined using the BCA Assay Kit (Thermo Scientific, Waltham, MA).

Western Blot Analysis

Cells were seeded at 1,500 cells/cm^2 and cultured for 10 days. Cells were then harvested and sonicated in 70 µl of lysis buffer (1 mM $NaHCO_3$, 2 mM PMSF, 1 mM Na orthovanadate, 5 mm EDTA and 1× protease inhibitor), and Western blotting was performed as previously described (Thi et al. 2010b). Briefly, protein samples were loaded onto 10 % SDS-PAGE gels for separation and electrophoretically transferred to nitrocellulose membranes. Membranes were probed with primary polyclonal antibodies against Cx43 (1:10,000, Sigma-Aldrich), $P2X_7R$ (1:1,000; Alomone Labs, Jerusalem, Israel) and Panx1 (mid, 1:100; Invitrogen) and monoclonal antibody against β-actin (1:25,000, Sigma-Aldrich), followed by incubation with the secondary antibody, horseradish peroxidase–conjugated anti-rabbit IgG or anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were detected using the Immobilon Western detection kit and exposed on the In Vivo FX PRO imaging system (Carestream, Rochester, NY).

YoPro-1 Dye-Uptake Analysis

MOB-C, 43KO-MOB-C and MC3T3-E1 cells were seeded at 2,000 cells/cm^2 and grown for 3 days on MatTek glass-bottomed dishes (MatTek, Ashland, MA). All dye-uptake experiments were performed in low divalent cation PBS (LDPBS, Ca^{2+}/Mg^{2+}-free), a condition that has been routinely used in experiments with connexin hemichannels and $P2X_7$Rs to maximize channel activation (Liu et al. 1996; Virginio et al. 1997; North and Surprenant 2000; Contreras et al. 2003; Jiang and Cherian 2003; Parpura et al. 2004; Cherian et al. 2005; Burra et al. 2010; Batra et al. 2012a). Cells were pretreated with the $P2X_7R$ blocker brilliant blue G (BBG, Sigma-Aldrich) and the connexin/pannexin blockers carbenoxolone (CBX, Sigma-Aldrich) and mefloquine (QU-024, Bioblocks, San Diego, CA) in serum-free α-MEM at 37 °C for 20 min prior to application of 5 µM YoPro-1 (Invitrogen) dye in LDPBS with or without the P2 receptor agonist ATP (Sigma-Aldrich) for 10 min. YoPro-1 dye was used because it is nonfluorescent in solution and fluoresces only when permeating the cells and interacting with nucleic acids. Three to four images per treatment were taken from each set of experiments using a Nikon (Melville, NY) Eclipse TE300 microscope and a Spot-RT digital camera (Diagnostic Instruments, Sterling Heights, MI) with fixed gain and exposure time.

Statistical Analysis

YoPro uptake was quantified as the number of YoPro-positive cells divided by the total number of cells, then multiplied by 100 (percent YoPro-positive cells), and counted using ImageJ software (NIH, Maryland, MD). Data were analyzed from three to four independent sets of experiments using Prism 5 software (GraphPad, San Diego, CA). Statistical differences between PGE_2 released amounts and YoPro-1 dye uptake were determined by one-way ANOVA, followed by Tukey's multiple comparison test. $P < 0.05$ was considered statistically significant.

Results

Participation of Cx43 Hemichannels and $P2X_7Rs$ in Fluid Shear Stress–Induced PGE_2 Release from Osteoblasts

Most previous studies on the role of Cx43 hemichannels in shear-induced ATP or PGE_2 release have relied on the use of compounds that do not pharmacologically discriminate Cx43 from Panx1-$P2X_7R$ (Cherian et al. 2005; Genetos et al. 2007). Therefore, we used Cx43-null or $P2X_7R$-null osteoblasts to specifically address each channel's role. As shown in Fig. 1, significant PGE_2 is released from MC3T3-E1 cells, primary osteoblasts (PMOB), primary Cx43-null osteoblasts (43KO-PMOB) and hTERT-immortalized wild-type (MOB-C) and Cx43-null (43KO-MOB-C) osteoblasts in response to pulsatile fluid shear stress (PFSS). In contrast, PFSS-induced PGE_2 release was completely absent in primary osteoblasts lacking $P2X_7R$ ($P2X_7$RKO-PMOB), as shown previously by another group (Li et al. 2005). PGE_2 released amounts under static conditions were not significantly different among all the cell types. This finding shows that under these conditions $P2X_7R$, rather than Cx43, plays a crucial role in shear-induced PGE_2 release from osteoblasts.

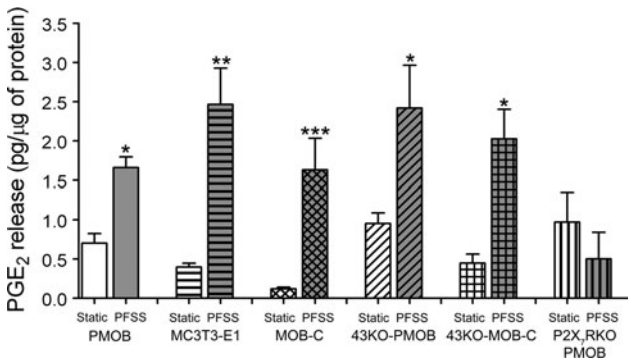


Fig. 1 Analysis of pulsatile fluid shear stress (*PFSS*)–induced PGE_2 release from MC3T3-E1 cells, primary osteoblast (*PMOB*), primary Cx43-null osteoblast (*43KO-PMOB*), $P2X_7R$-null osteoblasts (*$P2X_7$RKO-PMOB*) and hTERT-immortalized wild-type (*MOB-C*) and Cx43-null (*43KO-MOB-C*) osteoblasts. Supernatants from static and PFSS conditioned media were assayed for PGE_2 using the PGE_2 EIA kit. PGE_2 concentrations in the media were determined from standard curves obtained for each set of experiments, and the amount of PGE_2 release was normalized to respective cellular protein content. All data are presented as mean ± SEM, $n = 4$. P values were obtained using one-way ANOVA, followed by Tukey's multiple comparison test (***$P < 0.0005$, **$P < 0.005$, *$P < 0.05$ for static control vs. respective PFSS = 10 dyne/cm^2 at 1 Hz for 1 h)

To evaluate the extent to which the cell lines used in these studies express the channels of interest, we screened MC3T3-E1, MOB-C and 43KO-MOB-C cells for expression of Cx43, $P2X_7R$ and Panx1 using Western blot analysis. As shown in Fig. 2, all three lines expressed $P2X_7R$, as was expected from the well-established role of these receptors in bone resorption and mechanotransduction (Gallagher 2004; Li et al. 2005). In addition, Panx1 was found in all cell lines, consistent with its reported expression in primary osteoblasts (Penuela et al. 2007), and Cx43 was found in both MC3T3-E1 and MOB-C but not in 43KO-MOB-C (Thi et al. 2010b).

Cx43 Hemichannels Do Not Mediate Dye Uptake in Low Divalent Cation Solution

To study whether Cx43 was involved in dye uptake resulting from the mechanical stimulation induced by medium displacement in the imaging dish, we performed control basal level YoPro dye-uptake experiments in LDPBS, a condition reported to favor hemichannel opening (Contreras et al. 2003; Cherian et al. 2005; Burra et al. 2010; Batra et al. 2012a). Our results indicate that mechanically induced YoPro dye uptake in LDPBS solution was virtually identical in MC3T3-E1, MOB-C and 43KO-MOB-C cells and that this dye uptake was thus independent of whether Cx43 was present or absent (Fig. 3).

When we treated all three cell types with 1 mM ATP, YoPro uptake significantly increased (Fig. 4, solid gray bars), indicating that ATP could induce large pore formation in osteoblasts regardless of whether Cx43 was present or absent. Next, we examined the extent to which Cx43

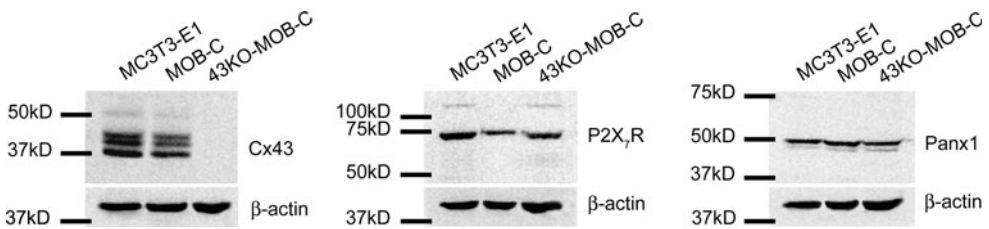


Fig. 2 Western blot assessment of Cx43, $P2X_7R$ and Panx1 expression levels in MC3T3-E1, MOB-C and 43KO-MOB-C cells. Equal amounts of protein from each cell type were used. Western blot analysis was performed using antibodies against Cx43, $P2X_7R$, Panx1 and β-actin. β-actin was used as a constitutively expressed protein for loading control

channel blockers inhibited dye uptake into MC3T3-E1, MOB-C and 43KO-MOB-C cells. For these studies, we used two compounds originally shown to block gap junction channels but subsequently found to be much more potent inhibitors of Panx1 channels at much lower concentrations. CBX is a widely used gap junction blocker, effective at concentrations of 100 μM and higher (Cherian et al. 2005; Batra et al. 2012a); we found a radical decrease in ATP-induced dye uptake not only in wild-type cells but also in Cx43-null cells, using a lower concentration that blocks Panx1 (20 μM) (see Bruzzone et al. 2005; Iglesias et al. 2008; Poornima et al. 2012) (Fig. 4, gray striped bars). This finding of blockade of dye uptake by low CBX concentration in both wild-type and Cx43-null osteoblast cells strongly suggests that Cx43 hemichannels do not play a critical role in large pore formation induced by ATP. This also suggests that a channel other than Cx43, likely Panx1, is responsible for the dye uptake.

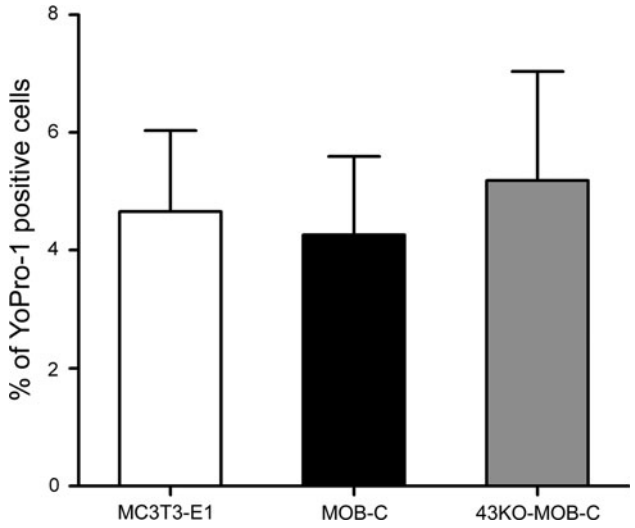


Fig. 3 Assessment of basal level YoPro-1 uptake resulting from the mechanical stimulation induced by medium displacement in the imaging dish with MC3T3-E1, MOB-C and 43KO-MOB-C cells. Cells were bathed in α-MEM at 37 °C for 20 min prior to 5 μM YoPro incubation for 10 min in low divalent cation PBS (*LDPBS*). Percent of YoPro-1-positive cells was calculated by normalizing dye-positive cells with total number of cells in each image field × 100. All data are presented as mean ± SEM, $n = 4$, for each cell type and compared using one-way ANOVA, followed by Tukey's multiple comparison test

To test the participation of Panx1 in ATP-induced pore formation, we also used mefloquine (MFQ). MFQ was originally shown to block gap junction channels [IC_{50} for Cx43–25 μM (Cruikshank et al. 2004)] but is now known to be a much more potent blocker for Panx1, being effective at submicromolar concentrations (Iglesias et al. 2009b). We used three low concentrations of MFQ (10, 50 and 90 nM) that were far below those with effects on Cx43 channels on other cell types (Cruikshank et al. 2004). We found that while MFQ only slightly inhibited basal dye influx in LDPBS (Fig. 5a–c, hatched bars), ATP-induced dye uptake was substantially reduced in MC3T3-E1 cells (Fig. 5a, gray hatched bars) and in both MOB-C and 43KO-MOB-C cells (Fig. 5c, gray hatched bars). In particular, ATP-induced dye uptake was completely eliminated in the presence of 90 nM MFQ in all cell lines. These results imply that Panx1, rather than Cx43, is the mechanosensitive channel being activated by ATP that provides the influx pathway for YoPro uptake.

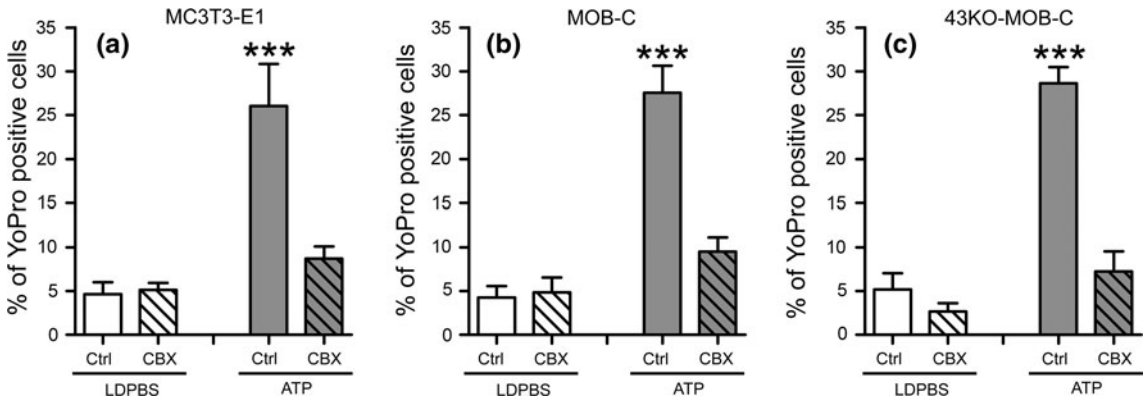
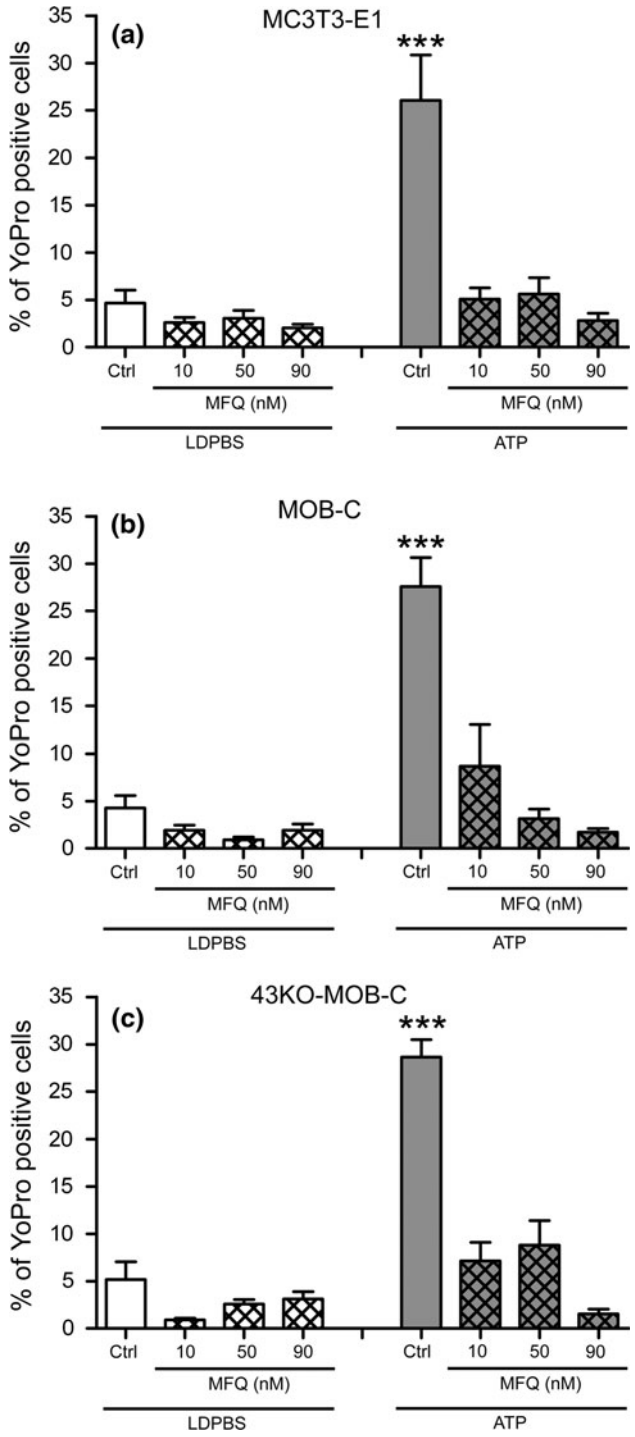


Fig. 4 Effects of carbenoxolone (*CBX*) treatment on YoPro-1 uptake induced either by mechanical stimulation caused by liquid displacement or by ATP application in **a** MC3T3-E1, **b** MOB-C and **c** 43KO-MOB-C osteoblasts. For these studies, cells were bathed in 5 μM YoPro-1 and the effects of 20 μM CBX were evaluated 20 min after treatment. For the ATP-stimulated group, 1 mM ATP was added 10 min after CBX. All experiments were performed in low divalent cation PBS (*LDPBS*). Percent of YoPro-1-positive cells was calculated by normalizing dye-positive cells with the total number of cells in each image field × 100. All data are presented as mean ± SEM, $n = 3$. *P* values were obtained using one-way ANOVA, followed by Tukey's multiple comparison test (***$P < 0.0005$, ATP-treated cells vs. all other treatments)



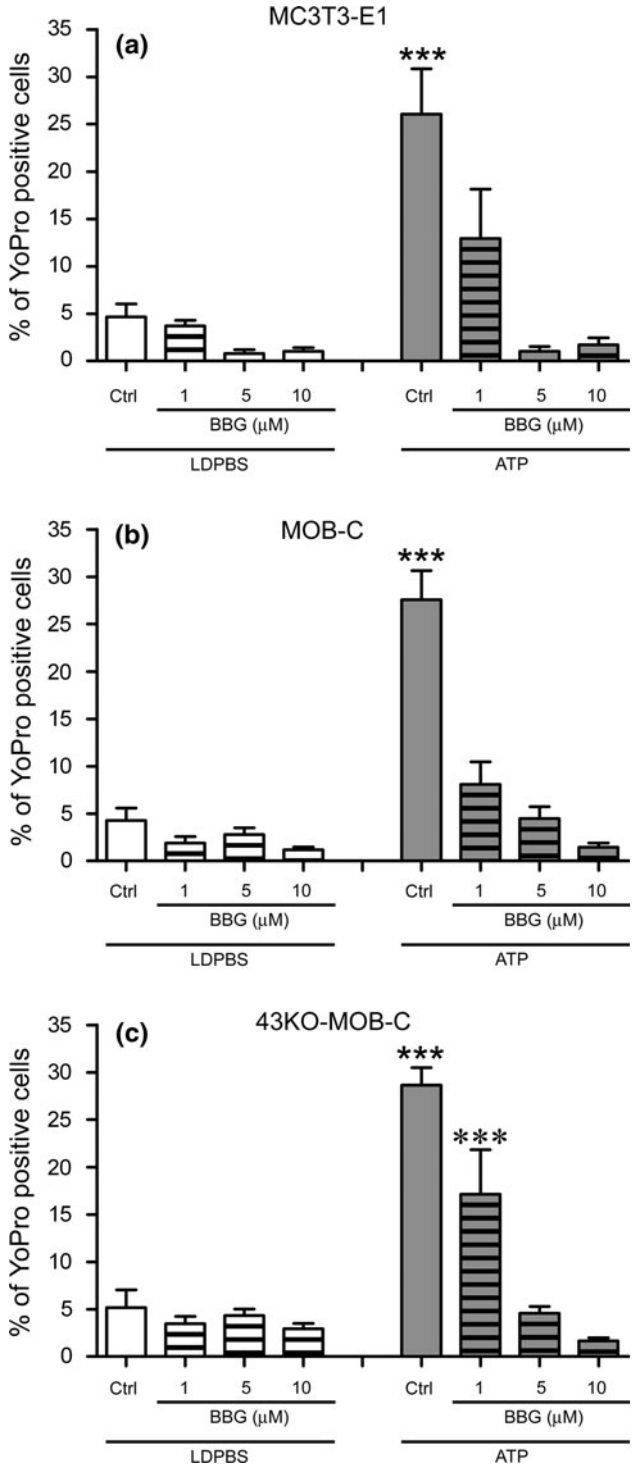
◀ **Fig. 5** Effects of mefloquine (*MFQ*) on YoPro-1 uptake induced either by mechanical stimulation caused by liquid displacement or by ATP application in **a** MC3T3-E1, **b** MOB-C and **c** 43KO-MOB-C osteoblasts. For these studies, cells were bathed in 5 μM YoPro-1 and the effects of three different concentrations of MFQ (10, 50 and 90 nM) were evaluated 20 min after treatment. For the ATP-stimulated group, 1 mM ATP was added 10 min after MFQ. All experiments were performed in low divalent cation PBS (*LDPBS*). Percent of YoPro-1-positive cells was calculated by normalizing dye-positive cells with the total number of cells in each image field × 100. All data are presented as mean ± SEM, $n = 3$. *P* values were obtained using one-way ANOVA, followed by Tukey's multiple comparison test (***$P < 0.0005$, ATP-treated cells vs. all other treatments)

Participation of the Panx1-P2X$_7$R Complex in ATP-Induced Dye Uptake

It has been shown that mice lacking P2X$_7$R have osteopenia in load-bearing bones, implying a critical role for P2X$_7$R as a key mediator in the skeletal response to mechanical loading (Ke et al. 2003). Moreover, ATP signaling through P2X$_7$R has been implicated in fluid shear stress–induced release of PGE_2 in bone cells (Li et al. 2005). There is accumulating evidence that P2X$_7$R functionally interacts with Panx1, providing a conduit for ATP-induced ATP release (Locovei et al. 2007; Iglesias et al. 2008). Therefore, to test the hypothesis that the P2X$_7$R-Panx1 complex in osteoblasts provides the pathway for ATP-induced dye influx, we tested the effects of BBG, a P2X$_7$R blocker. As shown in Fig. 6, all cell lines showed inhibition of ATP-induced dye uptake by BBG in a dose-dependent manner. At higher concentrations (5 and 10 μM) BBG completely abolished ATP-induced dye uptake in both wild-type osteoblastic cells and 43KO-MOB-C cells, demonstrating that P2X$_7$Rs play a role in such uptake. These collective findings strongly indicate that a pathway other than Cx43 hemichannels, likely the P2X$_7$R-Panx1 complex, participates in dye uptake under static conditions.

Discussion

There are several types of channels in osteoblasts and other cell types that are permeable to molecules as large as 1 kDa (Spray et al. 2006). The most prominent of those are channels formed by the gap junction protein Cx43 and the P2X$_7$R-Panx1 complex. Gap junction hemichannels have been proposed to underlie the large-conductance anion channel in skeletal myocytes and elsewhere (Blatz and Magleby 1983; Schwarze and Kolb 1984) and were thought to be responsible for permeabilization of J774 cells by high ATP concentration (Beyer and Steinberg 1991). This permeabilization by ATP now appears to be the result of P2X$_7$R activation in association with Panx1 (Pelegrin and Surprenant 2006; Locovei et al. 2007), and the permeability of these channels to fluorescent dyes has been shown to be very similar to that of gap junction channels (compare Flagg-Newton et al. 1979; Di Virgilio et al. 1996). Moreover, pharmacological blockade of connexins and Panx1 is achieved by most of the same agents. It is thus possible that roles attributed to one of these classes of molecules could be performed by the other.



◀ **Fig. 6** Effects of brilliant blue G (*BBG*) treatment on YoPro-1 uptake induced either by mechanical stimulation caused by liquid displacement or by ATP application in **a** MC3T3-E1, **b** MOB-C and **c** 43KO-MOB-C osteoblasts. For these studies, cells were bathed in 5 μM YoPro-1 and the effects of three different concentrations of BBG (1, 5 and 10 μM) were evaluated 20 min after treatment. For the ATP-stimulated group, 1 mM ATP was added 10 min after BBG. All experiments were performed in low divalent cation PBS (*LDPBS*). Percent of YoPro-1-positive cells was calculated by normalizing dye-positive cells with the total number of cells in each image field × 100. All data are presented as mean ± SEM, $n = 3$. *P* values were obtained using one-way ANOVA, followed by Tukey's multiple comparison test (***$P < 0.0005$, ATP-treated cells vs. all other treatments, ***$P < 0.0005$, 1 μM BBG vs. 1 μM BBG + ATP)

In bone cells, fluid shear stress induces the release of extracellular mechanosignaling molecules such as PGE_2, nitric oxide (NO), ATP and VEGF that are essential for bone homeostasis (Reich and Frangos 1993; Klein-Nulend et al. 1995; Genetos et al. 2005; Thi et al. 2010a). However, the cellular pathways that are involved in the release of these substances are not well characterized in bone cells. Studies with other cell types suggest that Cx43 hemichannels, Panx1 channels and the ionotropic purinergic $P2X_7$ receptor (Fig. 7) most likely assist in the release of these substances (Cherian et al. 2005; Li et al. 2005; Locovei et al. 2006).

Despite the existence of a small pool of unpaired Cx43 connexons ("hemichannels") on the unopposed cell surface (Dermietzel et al. 2003), there is little evidence for opening of these channels under physiological conditions. Nevertheless, most reports of functional hemichannels use as evidence dye-uptake measurements at normal resting potentials, with or without divalent cation chelation, and validation by use of gap junction channel blockers (Hofer and Dermietzel 1998; Stout et al. 2002; Contreras et al. 2003; Goodenough and Paul 2003). However, dye uptake mediated by the $P2X_7R$-Panx1 complex is Ca^{2+} and mechanosensitive, increased by membrane depolarization and even more sensitive to blockade by CBX than are gap junctions (Bao et al. 2004; Suadicani et al. 2012). Activation of $P2X_7Rs$ was shown to mediate ATP release in astrocytes (Anderson et al. 2004; Suadicani et al. 2006), and PGE_2 release from osteoblasts in response to fluid shear stress is absent in $P2X_7R$-null mice (Li et al. 2005). Studies on cells in which Panx1 expression was manipulated have shown that Panx1 provides the channel for $P2X_7R$-induced release of ATP (Locovei et al. 2007), which has been confirmed using the Panx1-null mouse (Suadicani et al. 2012). Perhaps most interesting of all, activation of Panx1 channels has been shown to be mechanosensitive (Bao et al. 2004). Therefore, although Cx43 hemichannels have been the focus of many studies in bone mechanotransduction (Jiang and Cherian 2003; Cherian et al. 2005; Genetos et al. 2007; Burra et al. 2010; Batra et al. 2012b), we here provide evidence for $P2X_7R$-Panx1 complex involvement in mechanically induced ATP release from osteoblasts (as illustrated in Fig. 7) and PGE_2 release via an unidentified pathway that does not require Cx43.

The combined use of pharmacological blockers and of osteoblasts lacking Cx43 or $P2X_7R$ in the studies described here has allowed discrimination of the specific role of each

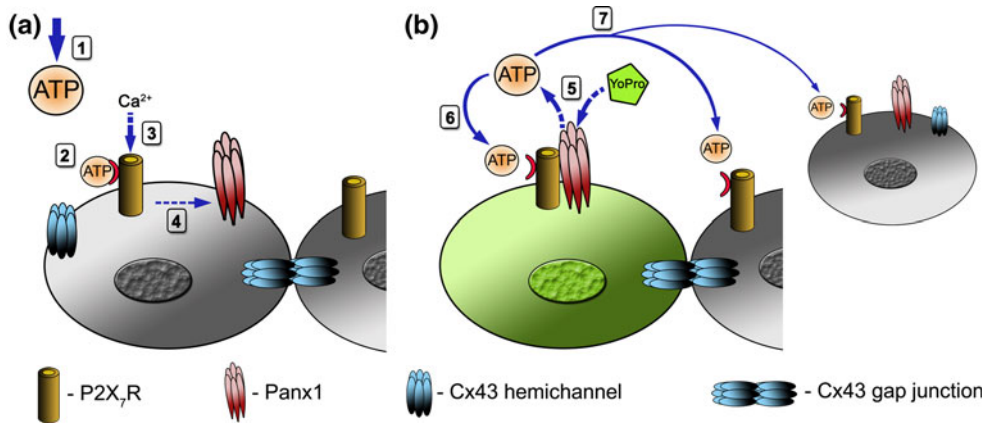


Fig. 7 Schematic diagram depicting the sequence of events triggered by ATP-induced activation of $P2X_7Rs$ in osteoblasts and the distribution of $P2X_7Rs$, Panx1 channels and Cx43 when forming gap junction channels and hemichannels. **a** Extracellular ATP (exogenously added or release from mechanically stimulated cells) diffuses (*1*), binds and activates ionotropic $P2X_7Rs$ (*2*), resulting in influx of Ca^{2+} (*3*) and $P2X_7R$ interaction with Panx1 channels (*4*). **b** P2X7 activation induces opening of Panx1 channels, providing a pathway for ATP efflux and for YoPro dye uptake (*5*). Autocrine $P2X_7R$ activation by released ATP triggers further ATP-induced ATP release from stimulated cells (*6*) and enhances paracrine signaling (*7*) to the neighboring cells. Gap junction channels formed of Cx43 also play a role in intercellular signaling through exchange of second messengers

of these molecular mediators of intercellular communication. While we find that mechanically induced PGE_2 release and ATP-induced dye uptake are not dependent on the presence or function of Cx43, further dissection of the roles of components of the $P2X_7R$-Panx1 complex await the use of Panx1-null osteoblastic cell lines, which are currently being generated in our laboratory.

Acknowledgments This work was supported by the National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Disease [Grants DK091466 (to M.M.T.), DK081435 (to S.O.S.)] and the National Institute of Arthritis and Musculoskeletal and Skin Diseases [Grant AR057139 (to D.C.S)].

References

Anderson CM, Bergher JP, Swanson RA (2004) ATP-induced ATP release from astrocytes. J Neurochem 88:246–256

Bao L, Locovei S, Dahl G (2004) Pannexin membrane channels are mechanosensitive conduits for ATP. FEBS Lett 572:65–68

Batra N, Burra S, Siller-Jackson AJ, Gu S, Xia X, Weber GF, Desimone D, Bonewald LF, Lafer EM, Sprague E, Schwartz MA, Jiang JX (2012a) Mechanical stress–activated integrin alpha5beta1 induces opening of connexin 43 hemichannels. Proc Natl Acad Sci USA 109:3359–3364

Batra N, Kar R, Jiang JX (2012b) Gap junctions and hemichannels in signal transmission, function and development of bone. Biochim Biophys Acta 1818:1909–1918

Beyer EC, Steinberg TH (1991) Evidence that the gap junction protein connexin-43 is the ATP-induced pore of mouse macrophages. J Biol Chem 266:7971–7974

Blatz AL, Magleby KL (1983) Single voltage-dependent chloride-selective channels of large conductance in cultured rat muscle. Biophys J 43:237–241

Bruzzone R, Barbe MT, Jakob NJ, Monyer H (2005) Pharmacological properties of homomeric and heteromeric pannexin hemichannels expressed in *Xenopus* oocytes. J Neurochem 92:1033–1043

Burra S, Nicolella DP, Francis WL, Freitas CJ, Mueschke NJ, Poole K, Jiang JX (2010) Dendritic processes of osteocytes are mechanotransducers that induce the opening of hemichannels. Proc Natl Acad Sci USA 107:13648–13653

Cherian PP, Siller-Jackson AJ, Gu S, Wang X, Bonewald LF, Sprague E, Jiang JX (2005) Mechanical strain opens connexin 43 hemichannels in osteocytes: a novel mechanism for the release of prostaglandin. Mol Biol Cell 16:3100–3106

Civitelli R (2008) Cell–cell communication in the osteoblast/osteocyte lineage. Arch Biochem Biophys 473:188–192

Contreras JE, Saez JC, Bukauskas FF, Bennett MV (2003) Gating and regulation of connexin 43 (Cx43) hemichannels. Proc Natl Acad Sci USA 100:11388–11393

Cruikshank SJ, Hopperstad M, Younger M, Connors BW, Spray DC, Srinivas M (2004) Potent block of Cx36 and Cx50 gap junction channels by mefloquine. Proc Natl Acad Sci USA 101:12364–12369

Dahl G, Locovei S (2006) Pannexin: to gap or not to gap, is that a question? IUBMB Life 58:409–419

Dermietzel R, Meier C, Bukauskas F, Spray DC (2003) Following tracks of hemichannels. Cell Commun Adhes 10:335–340

Di Virgilio F, Ferrari D, Falzoni S, Chiozzi P, Munerati M, Steinberg TH, Baricordi OR (1996) P2 purinoceptors in the immune system. Ciba Found Symp 198:290–305

Donahue HJ (2000) Gap junctions and biophysical regulation of bone cell differentiation. Bone 26:417–422

Flagg-Newton J, Simpson I, Loewenstein WR (1979) Permeability of the cell-to-cell membrane channels in mammalian cell junction. Science 205:404–407

Gallagher JA (2004) ATP P2 receptors and regulation of bone effector cells. J Musculoskelet Neuronal Interact 4:125–127

Genetos DC, Geist DJ, Liu D, Donahue HJ, Duncan RL (2005) Fluid shear-induced ATP secretion mediates prostaglandin release in MC3T3-E1 osteoblasts. J Bone Miner Res 20:41–49

Genetos DC, Kephart CJ, Zhang Y, Yellowley CE, Donahue HJ (2007) Oscillating fluid flow activation of gap junction hemichannels induces ATP release from MLO-Y4 osteocytes. J Cell Physiol 212:207–214

Goodenough DA, Paul DL (2003) Beyond the gap: functions of unpaired connexon channels. Nat Rev Mol Cell Biol 4:285–294

Hofer A, Dermietzel R (1998) Visualization and functional blocking of gap junction hemichannels (connexons) with antibodies against external loop domains in astrocytes. Glia 24:141–154

Iglesias R, Locovei S, Roque A, Alberto AP, Dahl G, Spray DC, Scemes E (2008) P2X7 receptor-pannexin1 complex: pharmacology and signaling. Am J Physiol Cell Physiol 295:C752–C760

Iglesias R, Dahl G, Qiu F, Spray DC, Scemes E (2009a) Pannexin 1: the molecular substrate of astrocyte "hemichannels.". J Neurosci 29:7092–7097

Iglesias R, Spray DC, Scemes E (2009b) Mefloquine blockade of pannexin1 currents: resolution of a conflict. Cell Commun Adhes 16:131–137

Jiang JX, Cherian PP (2003) Hemichannels formed by connexin 43 play an important role in the release of prostaglandin E_2 by osteocytes in response to mechanical strain. Cell Commun Adhes 10:259–264

Johnson RG, Sheridan JD (1971) Junctions between cancer cells in culture: ultrastructure and permeability. Science 174:717–719

Johnson RG, Herman WS, Preus DM (1973) Homocellular and heterocellular gap junctions in *Limulus*: a thin-section and freeze-fracture study. J Ultrastruct Res 43:298–312

Kar R, Batra N, Riquelme MA, Jiang JX (2012) Biological role of connexin intercellular channels and hemichannels. Arch Biochem Biophys 524:2–15

Ke HZ, Qi H, Weidema AF, Zhang Q, Panupinthu N, Crawford DT, Grasser WA, Paralkar VM, Li M, Audoly LP, Gabel CA, Jee WSS, Dixon SJ, Sims SM, Thompson DD (2003) Deletion of the P2X7 nucleotide receptor reveals its regulatory roles in bone formation and resorption. Mol Endocrinol 17:1356–1367

Klein-Nulend J, Semeins CM, Ajubi NE, Nijweide PJ, Burger EH (1995) Pulsating fluid flow increases nitric oxide (NO) synthesis by osteocytes but not periosteal fibroblasts—correlation with prostaglandin upregulation. Biochem Biophys Res Commun 217:640–648

Lecanda F, Warlow PM, Sheikh S, Furlan F, Steinberg TH, Civitelli R (2000) Connexin43 deficiency causes delayed ossification, craniofacial abnormalities, and osteoblast dysfunction. J Cell Biol 151:931–944

Li J, Liu D, Ke HZ, Duncan RL, Turner CH (2005) The P2X7 nucleotide receptor mediates skeletal mechanotransduction. J Biol Chem 280:42952–42959

Liu TF, Li HY, Atkinson MM, Johnson RG (1996) Comparison of lucifer yellow leakage and cell-to-cell transfer following intracellular injection in normal and antisense Novikoff cells under treatment with low extracellular Ca^{2+}. Methods Find Exp Clin Pharmacol 18:493–497

Locovei S, Bao L, Dahl G (2006) Pannexin 1 in erythrocytes: function without a gap. Proc Natl Acad Sci USA 103:7655–7659

Locovei S, Scemes E, Qiu F, Spray DC, Dahl G (2007) Pannexin1 is part of the pore forming unit of the P2X7 receptor death complex. FEBS Lett 581:483–488

MacVicar BA, Thompson RJ (2010) Non-junction functions of pannexin-1 channels. Trends Neurosci 33:93–102

Minkoff R, Rundus VR, Parker SB, Hertzberg EL, Laing JG, Beyer EC (1994) Gap junction proteins exhibit early and specific expression during intramembranous bone formation in the developing chick mandible. Anat Embryol (Berl) 190:231–241

North RA, Surprenant A (2000) Pharmacology of cloned P2X receptors. Annu Rev Pharmacol Toxicol 40:563–580

Parpura V, Scemes E, Spray DC (2004) Mechanisms of glutamate release from astrocytes: gap junction "hemichannels," purinergic receptors and exocytotic release. Neurochem Int 45:259–264

Pelegrin P, Surprenant A (2006) Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. EMBO J 25:5071–5082

Penuela S, Bhalla R, Gong XQ, Cowan KN, Celetti SJ, Cowan BJ, Bai D, Shao Q, Laird DW (2007) Pannexin 1 and pannexin 3 are glycoproteins that exhibit many distinct characteristics from the connexin family of gap junction proteins. J Cell Sci 120: 3772–3783

Poornima V, Madhupriya M, Kootar S, Sujatha G, Kumar A, Bera AK (2012) P2X7 receptor-pannexin 1 hemichannel association: effect of extracellular calcium on membrane permeabilization. J Mol Neurosci 46:585–594

Reaume AG, de Sousa PA, Kulkarni S, Langille BL, Zhu D, Davies TC, Juneja SC, Kidder GM, Rossant J (1995) Cardiac malformation in neonatal mice lacking connexin43. Science 267: 1831–1834

Reich KM, Frangos JA (1993) Protein kinase C mediates flow-induced prostaglandin E_2 production in osteoblasts. Calcif Tissue Int 52:62–66

Romanello M, D'Andrea P (2001) Dual mechanism of intercellular communication in HOBIT osteoblastic cells: a role for gap-junctional hemichannels. J Bone Miner Res 16:1465–1476

Scemes E, Suadicani SO, Dahl G, Spray DC (2007) Connexin and pannexin mediated cell–cell communication. Neuron Glia Biol 3:199–208

Schiller PC, D'Ippolito G, Balkan W, Roos BA, Howard GA (2001) Gap-junctional communication is required for the maturation process of osteoblastic cells in culture. Bone 28:362–369

Schwarze W, Kolb HA (1984) Voltage-dependent kinetics of an anionic channel of large unit conductance in macrophages and myotube membranes. Pflugers Arch 402:281–291

Silverman WR, de Rivero Vaccari JP, Locovei S, Qiu F, Carlsson SK, Scemes E, Keane RW, Dahl G (2009) The pannexin 1 channel activates the inflammasome in neurons and astrocytes. J Biol Chem 284:18143–18151

Sosinsky GE, Boassa D, Dermietzel R, Duffy HS, Laird DW, MacVicar B, Naus CC, Penuela S, Scemes E, Spray DC, Thompson RJ, Zhao HB, Dahl G (2011) Pannexin channels are not gap junction hemichannels. Channels (Austin) 5:193–197

Spray DC, Ye ZC, Ransom BR (2006) Functional connexin "hemichannels": a critical appraisal. Glia 54:758–773

Stout CE, Costantin JL, Naus CC, Charles AC (2002) Intercellular calcium signaling in astrocytes via ATP release through connexin hemichannels. J Biol Chem 277:10482–10488

Suadicani SO, Brosnan CF, Scemes E (2006) P2X7 receptors mediate ATP release and amplification of astrocytic intercellular Ca^{2+} signaling. J Neurosci 26:1378–1385

Suadicani SO, Iglesias R, Wang J, Dahl G, Spray DC, Scemes E (2012) ATP signaling is deficient in cultured pannexin1-null mouse astrocytes. Glia 60:1106–1116

Thi MM, Suadicani SO, Spray DC (2010a) Fluid flow-induced soluble vascular endothelial growth factor isoforms regulate actin adaptation in osteoblasts. J Biol Chem 285:30931–30941

Thi MM, Urban-Maldonado M, Spray DC, Suadicani SO (2010b) Characterization of hTERT-immortalized osteoblast cell lines generated from wild-type and connexin43-null mouse calvaria. Am J Physiol Cell Physiol 299:C994–C1006

Virginio C, Church D, North RA, Surprenant A (1997) Effects of divalent cations, protons and calmidazolium at the rat P2X7 receptor. Neuropharmacology 36:1285–1294