Gap Junctional Intercellular Communication in Bone

Henry J. Donahue
The Pennsylvania State University College of Medicine

Monique A. Vander Molen
State University of New York

Clare E. Yellowley
The Pennsylvania State University College of Medicine

Zhongyong Li
The Pennsylvania State University College of Medicine

Utah State University
MERRILL-CAZIER LIBRARY

GAP JUNCTIONAL INTERCELLULAR COMMUNICATION IN BONE

Henry J. Donahue[1*], Monique A. Vander Molen[2], Clare E. Yellowley[1] and Zhongyong Li[1]

[1]Musculoskeletal Research Laboratory, Departments of Orthopaedics and Rehabilitation and Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, Hershey, PA, and [2]Department of Orthopaedics, State University of New York, Stony Brook, NY

Abstract

Gap junctional intercellular communication has been demonstrated in bone cells and may contribute to the mechanism by which osteoblasts integrate and amplify extracellular signals, both chemical (hormonal) and biophysical (electrical and mechanical). Connexin 43 (Cx43) is the predominant gap junction protein expressed by bone cells. Experiments with osteoblastic cells in which Cx43 expression was diminished by antisense transfection demonstrate that cell-to-cell coupling in osteoblastic ROS 17/2.8 cells is via gap junctions composed of Cx43. Cellular networks of these coupling deficient clones are dramatically less responsive to parathyroid hormone (PTH) suggesting that coupling contributes to hormonal responsiveness. Furthermore, PTH *per se* can upregulate cell-to-cell communication in these networks. Membrane deformation-induced Ca^{2+} signals propagate from the deformed cell to neighboring undeformed cells. This phenomenon is blocked by octanol, a gap junction uncoupler. Physiologically relevant electric fields, i.e., induced by mechanical load, stimulate alkaline phosphatase activity in ROS 17/2.8 cells, but this response is greatly reduced in coupling deficient Cx43 antisense transfectants. Furthermore, electric fields *per se* regulate Cx43 expression in osteoblastic cells. Gap junctional intercellular communication appears critical for the regulation of osteoblastic behavior and thus bone metabolism by extracellular signals.

Key Words: Gap junctions, bone, osteoblast, osteocyte, connexin, parathyroid hormone (PTH), electric fields, mechanochemical transduction, intercellular communication, biophysical signal transduction.

*Address for correspondence:
Henry J. Donahue
Musculoskeletal Research Laboratory
Department of Orthopaedics and Rehabilitation
The Pennsylvania State University College of Medicine
P.O. Box 850, Hershey, PA 17033-0850

Telephone Number: (717) 531-4819
FAX Number: (717) 531-7583

Introduction

Gap junctions are membrane spanning channels which facilitate intercellular communication by allowing passage of small molecules (< 1 kD), such as calcium ion (Ca^{2+}), inositol phosphates and cyclic nucleotides, from one cell to another. Each gap junction is comprised of two hexameres termed connexons each of which in turn are comprised of 6 subunits termed connexins (Cx). At least twelve mammalian connexins have been identified and classified according to their derived molecular weight. The most widely expressed of these include Cx26 and Cx32, both originally isolated from rat liver [39, 60], and Cx43 from rat heart [5, 6]. Morphological and ultrastructural studies reveal that gap junctions directly connect (exist between) adjoining osteoblasts and osteocytes [20, 29] but their role in bone function is unclear. It has been suggested that gap junctional intercellular communication facilitates the integration and amplification of extracellular signals critical to the regulation of bone modeling and remodeling [21, 31]. Unfortunately, little experimental evidence exists which directly supports this concept. However, recent advances in the cell and molecular biology of gap junctions provide support for the idea that cell-to-cell communication contributes to signal transduction in bone.

There are several possible functions for gap junctional intercellular communication in bone. First, cell coupling could contribute to cell differentiation, tissue development and morphogenesis as is the case in other tissue systems [33, 42, 55, 59]. A second possibility is that gap junctions facilitate the transmission of extracellular signals, such as hormones, from the periphery where bone cells are in close proximity to blood vessels, to deeper cortical areas not exposed to blood flow. Likewise, cell coupling may facilitate transmission of osteogenic biophysical signals from one area of bone to another. Another possibility is that gap junctional coupling contributes to the coordinated responses of cellular networks to extracellular signals.

The purpose of this brief review is to survey recent data which support a role for gap junction intercellular

communication in signal integration and amplification within bone. Specifically, the contribution of cell-to-cell coupling to hormonal responsiveness and biophysical signal transduction will be discussed.

Characterization of Gap Junctions in Bone

The earliest evidence suggesting functional gap junctions in bone was provided by Jeansonne et al. [28]. These investigators reported that when rat calvarial subperiosteal osteoblasts were injected with a 0.5 kD fluorescent dye the dye spread to neighboring osteoblastic cells. Furthermore, passage of current from one cell to another resulted in a membrane potential change in the second cell. While this was the first study to suggest cell-to-cell coupling between osteoblastic cells, it is unclear whether this coupling was gap junction mediated. Indeed, the spread of ionic current may have been independent of gap junctions. Furthermore, these investigators also demonstrated that parathyroid hormone conjugated fluorescein, a molecule much larger than the 1 kD limit for gap junction channels, passed from cell-to-cell. These results suggest that the cell-to-cell communication demonstrated by these investigators may have been by means other than gap junctional channels. Subsequently, electron microscopy demonstrated gap junctions between adjacent osteoblasts and between osteoblasts and osteocytes [20]. No evidence was found for gap junctions between osteoclasts and either osteocytes or osteoblasts. These early electromicrographic studies have been confirmed by several investigators [1, 29, 38, 48] and, while they did not characterize the structure or function of gap junctions in bone, strongly suggested a role for gap junctions in bone metabolism.

Recent advances in the molecular biology of gap junctions have allowed the identification of specific gap junction proteins expressed in bone and analysis of their functional characteristics. Schirrmacher et al. [47] presented the first evidence that a specific connexin, connexin 43 (Cx43), is expressed in osteoblastic cells. Cx43 was first identified in rat heart [6] and is perhaps the most widely distributed connexin being found in several tissue types including myometrium, testis, lens epithelium, endothelium [4], and cartilage [17]. Schirrmacher et al. [46] also demonstrated that bovine osteoblasts were both metabolically and electrically coupled, i.e., communicate with one another via gap junctions. These findings were subsequently confirmed by several independent groups and taken together suggest that Cx43 may be the predominant functional gap junction protein expressed in human and other mammalian bone cells in vitro [9, 11, 19, 45, 58]. However, it is not clear that Cx43 is the predominant gap junction expressed in vivo since gap junction expression in vitro does not necessarily parallel expression in vivo.

The predominance of Cx43 in osteoblastic cells and the observation that osteoblasts are metabolically and electrically coupled is strong indirect evidence that they are coupled via gap junctions composed of Cx43. More direct evidence that at least one osteoblastic cell line, ROS 17/2.8, is coupled via gap junctions composed of Cx43 comes from studies characterizing functional coupling in ROS 17/2.8 cells expressing an antisense cDNA construct of Cx43 [53]. In these studies, ROS 17/2.8 cells were transfected with either a plasmid encoding an antisense construct of Cx43 and neomycin resistance or a control plasmid encoding neomycin resistance only. Two clones were isolated which expressed dramatically reduced expression of Cx43 mRNA and protein and reduced functional coupling (Fig. 1). However, these cells maintained other phenotypic characteristics of osteoblastic cells. These results not only provide strong direct evidence that an osteoblastic cell line is coupled via gap junctions composed of Cx43, but also provide a useful model to examine the role of cell coupling in osteoblastic behavior.

While Cx43 may be the predominant gap junction protein found in bone cells in vitro, Cx45, originally identified in heart and found in endometrium and a few other tissues, has been detected in a rat osteoblastic osteosarcoma cell line, UMR-106 [50], and an SV-40 transfected human fetal osteoblastic cell line, hFOB (Donahue et al., unpublished data). Interestingly, the Cx45 containing gap junction channels in UMR-106 cells are less permeable to Lucifer yellow, a dye which passes from cell-to-cell via gap junctions, than Cx43 containing channels in other osteoblastic cell lines such as ROS 17/2.8 [50]. Furthermore, when ROS 17/2.8 cells are transfected with a gene encoding Cx45 their permeability to Lucifer yellow decreases. Thus, osteoblastic cells may have the potential to regulate cell coupling by turning different connexins on or off.

Considerable data are accumulating which characterize gap junctions in osteoblastic networks, however much less information is available regarding gap junction expression in other bone cells. While Cx43 immunoreactivity has been demonstrated between adjacent osteocytes in situ [29], and in bone formed in vitro [24] and Cx43 mRNA within osteocytes has been identified in vivo by use of reverse transcriptase linked to polymerase chain reaction (RT-PCR) [34] there is no evidence that osteocytes are functionally coupled in vivo or in vitro. This is an extremely important issue since cell-to-cell communication within osteocytic networks has been postulated as a critical component of

extracellular signal transduction, integration and amplification in bone [15, 21, 31]. There is also little information regarding expression of gap junction proteins by osteoclasts. Cx 43 immunoreactivity has been identified between some osteoclasts in two week old rats [29] but whether these constitute functional gap junctions was not examined.

To summarize, osteoblastic cells communicate with one another via membrane channels composed of gap junctions. Additionally, the predominant gap junction protein expressed by bone cells *in vitro* appears to be Cx43.

Gap Junctions and Hormonal Responsiveness

Evidence that coupling coordinates the response of cell networks to hormones comes from studies demonstrating that pharmacological inhibitors of intercellular coupling inhibit adrenocorticotropic hormone-induced steroidogenesis in adrenal cells [37], bombesin-stimulated cytosolic calcium oscillations in pancreatic acini [49], the secretory effect of thyrotrophic releasing hormone on pituitary cells [22], and alpha$_1$ adrenergic receptor agonist-stimulated contractions in smooth muscle cells [10]. These studies suggest that cell-to-cell communication is critical for hormonal responsiveness in many tissue systems. However, these studies are difficult to interpret since the agents used to inhibit gap junctional coupling, in most cases lipophilic long chain alcohols, may have many non-specific effects on cells [2]. Therefore, we have used a more direct and specific approach to assess the role of intercellular communication in the ability of cell ensembles to respond to extracellular signals. Utilizing cx 43 deficient clones we have examined the role of cell-to-cell communication in hormonal responsiveness in bone cell networks [53].

To obtain Cx43 deficient clones osteoblastic ROS 17/2.8 cells were transfected with a plasmid containing an antisense cDNA construct to rat Cx43. Control transfection did not alter cell-to-cell coupling nor Cx43 mRNA or protein expression relative to nontransfected ROS 17/2.8 cells. In contrast, stable transfection with an antisense Cx43 cDNA resulted in two clones, RCx4 and RCx16, which displayed significant decreases in Cx43 mRNA (almost 100% reduction) and protein (greater than 83% reduction) expression and were dramatically deficient in cell-to-cell coupling. Phenotypically, all transfectants retained osteoblastic characteristics.

As shown in Figure 2, the cyclic AMP (cAMP) response to parathyroid hormone (PTH), a potent regulator of bone cell metabolism, was dramatically reduced in two Cx43 deficient, and therefore coupling
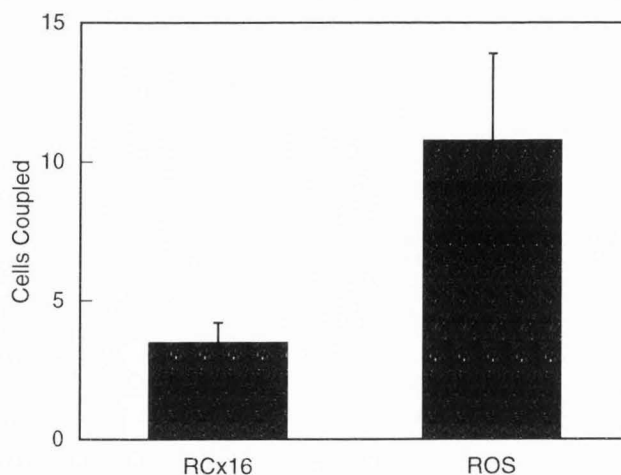

Figure 1. Cell coupling in Cx43 antisense transfected clones. ROS 17/2.8 cells were transfected with a plasmid encoding Cx43 cDNA in an antisense orientation. Functional coupling in one of the clones, RCx16, was then quantified, relative to ROS 17/2.8, by dye transfer. Individual cells were loaded with Lucifer yellow (molecular weight = 457 D), a fluorescent dye which passes through gap junctions, and the number of neighboring cells taking up dye within three minutes recorded. Each bar represents the mean ($\pm$ standard error of mean, SE) number of adjacent cells which take up dye within three minutes. This number was significantly lower (p < 0.05) in RCx16 as compared to ROS 17/2.8 reflecting a reduction in coupling capacity (n = 10-15 cells/group). These data confirm previous results [53] and suggest that Cx43 antisense transfection is quite stable.

----

deficient, clones relative to normal ROS 17/2.8 cells and control transfectants. Furthermore, the attenuation of PTH-stimulated cAMP accumulation in antisense transfected clones was not due to an effect of transfection on adenylate cyclase activity, or PTH receptor expression, availability or binding kinetics [53]. These data strongly suggest that cell coupling contributes to PTH responsiveness in osteoblastic cell networks.

This finding has important implications in bone tissue where the responsiveness of individual cells to a given hormonal signal can be quite heterogenous. For instance, Civitelli *et al.* [12] have shown that among individual osteoblasts the response to PTH is heterogeneous such that only 30% of osteoblastic cells respond to maximal doses of PTH (10^{-7} M) on an individual basis. Thus, in a population of cells exhibiting a heterogeneous response to a hormone on an individual basis gap junctions may act to amplify the effects of receptor activation of a single cell by
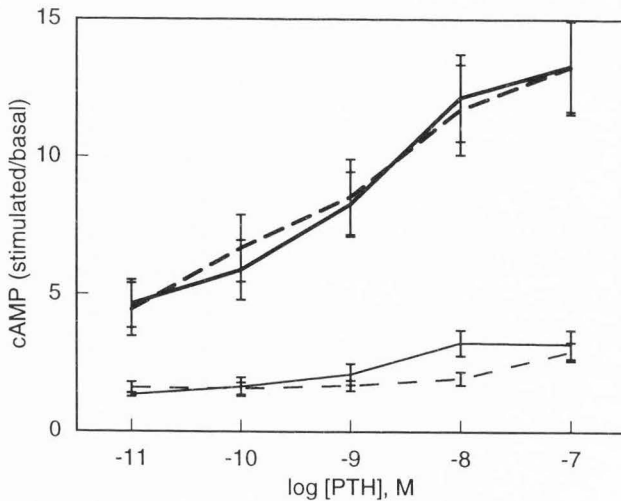
Figure 2. PTH-stimulated cAMP response in osteoblastic cells. Intracellular cAMP responses were measured by RIA after 15 minute exposures, at 37°C, to 10^{-7} - 10^{-11} M rPTH(1-34). Cells were plated at 50,000 cells/cm^2 and grown for 60 hours. PTH stimulated cAMP accumulation in a concentration-dependent manner in both ROS (thick line) and control transfectants (bG; thick dash). However, PTH-stimulated cAMP accumulation in RCx16 (thin dash) and RCx4 (thick dash), two Cx43 antisense transfected clones was dramatically attenuated. The peak cAMP response to 10^{-7} M rPTH(1-34) in RCx4 and RCx16 was only 26.2% and 21.9% that of ROS, respectively. All data represent mean ± SE of 3-6 experiments performed in duplicate. *: significantly different from either ROS or bG, p < 0.05. Adapted from Vander Molen et al. [53].

----

permitting the spread of second messengers to adjacent cells that are not directly activated by the agonist [10], in this case PTH. In this manner, the net cell ensemble response would be greater than the sum of individual responding cells.

Not only is cell-to-cell communication critical for hormonal responsiveness in osteoblastic networks but hormones, in turn, can regulate cell-to-cell communication. Schiller et al. [45] demonstrated that PTH can increase Cx43 gene expression and cell coupling in UMR-106 and rat calvarial cells. Furthermore, these effects were mimicked by forskolin and dbcAMP suggesting that PTH regulates cell-to-cell communication partly by a cAMP-dependent mechanism. PTH also stimulates, in a concentration-dependent manner, cell-to-cell communication in osteoblastic cells isolated from rat long bone [19] (Fig. 3). This effect is blocked by PTH (3-34), an amino truncated analog of
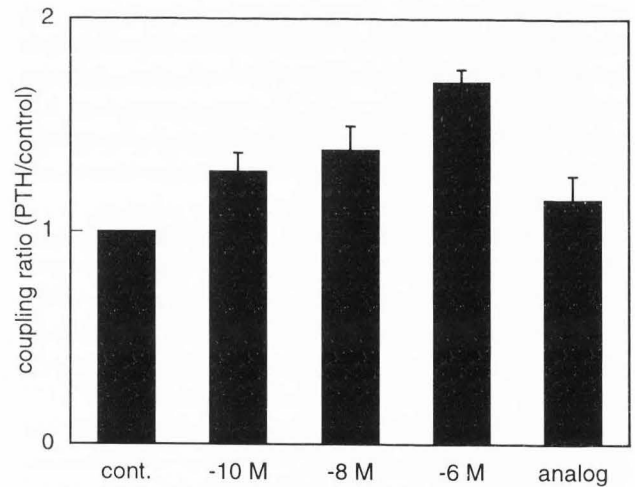

Figure 3. Concentration-dependent effect of PTH on intercellular communication in ROB cells. Cells were exposed to 10^{-10} M to 10^{-6} M rPTH-(1-34) or 10^{-6} M rPTH-(1-34) and 10^{-6} M PTH-(3-34) for one hour prior to being loaded with carboxyfluorescein and the percentage of cells coupled calculated. Values are the ratios of proportion of cells coupled in the presence of PTH to the proportion coupled in the presence of vehicle control (± SE of proportion). Control = vehicle control, values = log [PTH] ; analog = PTH-(1-34) plus PTH-(3-34). *: Significantly greater than vehicle control, p < 0.05. The total number of cells loaded for each group was: control = 53; -10 M = 28; -8 M = 15; -6 M = 22; analog = 39. From Donahue et al. [19] (reproduced with permission).

PTH which blocks PTH-simulated cAMP accumulation [16], supporting the concept that PTH stimulates cell coupling in osteoblasts via a cAMP-dependent mechanism. The implication from these findings is that any cell process dependent on cell-to-cell communication, i.e., differentiation, morphogenesis and signal transduction, may in turn be modulated by PTH.

Other hormones and extracellular factors have been shown to increase Cx43 function and expression in osteoblastic networks. For instance, retinoic acid and transforming growth factor ß, have been shown to increase expression of Cx43 protein and mRNA and increase coupling in SV-40 transformed human fetal osteoblastic cells, whereas 1,25 (OH)$_2$ vitamin D was without effect on these parameters. While the mechanism by which these factors affect coupling was not investigated, it is unlikely that it was through activation of adenylate cyclase activity since both retinoic acid [3] and transforming growth factor ß [30] inhibit PTH-stimulated cAMP accumulation. Studies are needed to determine the mechanism by which these

factors affect cell-to-cell coupling.

In summary, not only is cell-to-cell communication critical to hormonal responsiveness, at least as regards PTH, but it is also clear that PTH regulates cell-to-cell communication. Thus a feedback loop exists wherein PTH may act to amplify the effectiveness of its own signal. While such positive feedback loops are rare in physiological systems, they are not without precedent. Indeed, in the nervous system, where signal integration and amplification is the goal, action potentials are propagated via positive feedback. It is tempting to speculate that the osteoblastic/osteocytic network, defined by gap junctional intercellular communication and regulated by PTH, provides a positive feedback loop to function in signal integration and amplification as is the case in the nervous system. Interestingly, a role for positive feedback in bone remodeling has already been promulgated [51].

Gap Junctions and Biophysical Signal Transduction

One way cell coupling may contribute to biophysical signal transduction is by transmitting signals from individual cells which perceive a biophysical signal to those that do not. This is demonstrated in Figure 4. In this experiment the membrane of a single cell, in this case an immortalized human fetal osteoblastic cell (hFOB) [26], is deformed by a micropipette attached to a micromanipulator. This results in a transient increase in the concentration of cytosolic Ca^{2+}, a ubiquitous second messenger molecule, in the deformed cell which propagates to non-deformed cells with which it is in direct physical contact but which are not themselves deformed. When this experiment is repeated in the presence of octanol, a blocker of gap junctional coupling, cytosolic Ca^{2+} concentration increases in the deformed cell but not in the undeformed neighbors. This phenomenon has also been demonstrated in primary cultures of rat osteoblastic cells and in osteosarcoma cells [56]. While these findings suggest that gap junctional intercellular communication can facilitate the propagation of a mechanically-induced signal, they must be interpreted with caution since the mechanical signal used is rather non-physiological.

The molecule which is responsible for mechanically induced Ca^{2+} signal propagation is unknown. In a series of elegant experiences Sanderson *et al.* [44] demonstrated that mechanically-induced Ca^{2+} signal propagation occurred in the presence or absence of extracellular Ca^{2+}. However, in the absence of extracellular Ca^{2+} the cell which was mechanically perturbed did not demonstrate an increase in $[Ca^{2+}]$. Furthermore, injection of inositol trisphosphate (IP$_3$) evoked Ca^{2+} signal propagation and heparin, which
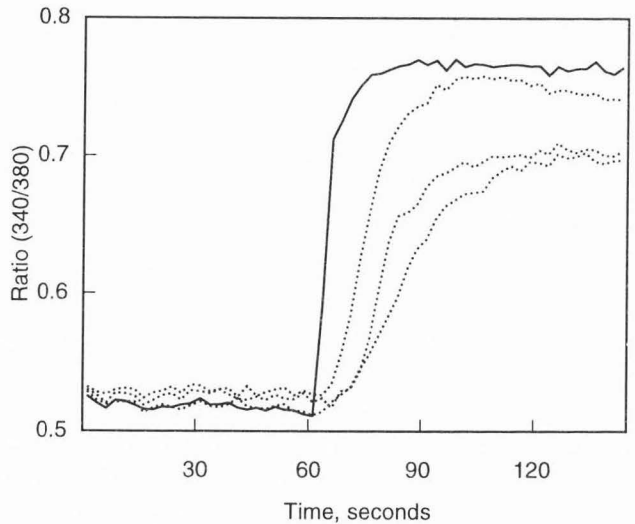

Figure 4. Mechano-chemical signal propagation in osteoblastic networks. Osteoblastic hFOB cells were loaded with the fluorescent Ca^{2+} indicator fura-2AM. The ratio of fluorescent intensity at excitation wavelengths of 340 and 380 nm reflects the intracellular Ca^{2+} ion concentration ($[Ca^{2+}]_i$). In this experiment a single cell membrane is deformed at 60 seconds resulting in an increase in $[Ca^{2+}]_i$. This Ca^{2+} signal then propagates to neighboring cells. A similar response was seen in 12 of 15 individual cells subjected to membrane deformation. However, when these experiments were repeated in the presence of octanol, an inhibitor of gap junctional coupling, Ca^{2+} signal propagation from deformed cells to undeformed cells occurred in only 2 of 19 individual cells. This demonstrates that a mechanically induced intracellular signal can propagate from cell-to-cell via gap junctions.

----

blocks IP$_3$-stimulated release of Ca^{2+} from intracellular stores, blocked mechanically-induced Ca^{2+} signal propagation [7]. Additionally, the phospholipase C inhibitor aminosteroid U73122, which blocks synthesis of IP$_3$, blocks Ca^{2+} signal propagation without affecting $[Ca^{2+}]_i$ in the mechanically perturbed cell [25]. Taken together these data strongly suggest that the mechanically-induced Ca^{2+} signal is propagated via IP$_3$ passage through gap junctions (for review see, Sanderson *et al.* [43]). Whether this is also the case in bone cells is unclear since Xia and Ferrier [56] did not see propagation of mechanically-induced Ca^{2+} signals in the absence of extracellular Ca^{2+} in primary rat calvaria osteoblasts and ROS 17/2.8 cells. Thus, mechanically-induced Ca^{2+} signal propagation in bone cells may be fundamentally different than in epithelial cells.

Another way in which cell coupling could facilitate biophysical signal transduction is by sensitizing, by

means of amplification, osteoblastic or osteocytic networks to threshold level biophysical signals such as endogenously produced electric fields. Low amplitude electric fields, on the order of $1-10\mu V/cm$ [35], can be induced by functional loading of bone via piezoelectric effects, streaming potentials, muscle cell activity or a combination of these [23, 27, 32]. While current evidence suggests that such weak electric fields can alter bone cell activity both *in vivo* [41] and *in vitro* [36] the mechanism by which this occurs is not known. Indeed, controversy regarding the validity of weak field responses has risen largely because of the theoretical objection that the very small field magnitudes reported to have effects appear to be too low to overcome noise inherent to the cell [54]. However, Cooper [13] and Pilla *et al.* [40] have presented theoretical arguments based on electrotonic cable theory which suggest that cellular networks coupled metabolically or electronically via gap junction channels are more sensitive to electric fields. Electrotonic cable theory predicts that the change in membrane potential induced by an electric field is proportional to the product of field intensity and cell radius. Therefore, very large cells or large networks of smaller cells, coupled via gap junctions into a metabolically contiguous network, will serve as an endogenous amplifier and thus be more sensitive to electric fields. We have utilized our Cx43 deficient clones to examine the role of cell coupling in bone cell responsiveness to electric fields.

Low level electromagnetic fields ($6\mu V/cm$) were previously shown to inhibit proliferation and stimulate alkaline phosphatase activity in ROS 17/2.8 cells [36]. Interestingly, this effect was demonstrated to be cell density-dependent suggesting a role for cell-to-cell communication. These experiments were repeated using coupling deficient Cx43 antisense transfected clones. Preliminary results [52] suggest that while a weak electric field increases alkaline phosphatase activity in well coupled ROS 17/2.8 cells, the same field fails to do so in coupling deficient cells. These findings support the concept that cell-to-cell communication contributes to the mechanism by which mechanical load-induced electric fields are detected by bone cell networks.

We have also examined the effect of electric fields *per se* on gap junctions in osteoblastic cell networks. Previous studies [14] suggest that electric fields increase gap junctional intercellular communication in lateral giant neurons of crayfish. However, in preliminary results [18], we found that electric fields decreased Cx43 mRNA and protein expression in ROS 17/2.8 cells. This would appear inconsistent with an upregulation of cell coupling by electric fields. However, these results are preliminary and the effect of electric fields on functional coupling in bone cells has not been investigated.

In summary, gap junctional coupling may contribute to biophysical signal transduction by facilitating propagation of mechanically-induced signals from cells that sense the signal to those which do not. Furthermore, both theoretical arguments and experimental results strongly suggest a role for cell coupling in sensitizing osteoblastic networks to weak electric fields such as may be induced by mechanical loading of bone. Whether osteocytes, the cells best situated to detect biophysical signals in bone, utilize cell coupling in this manner is yet to be determined.

Conclusions

Considerable evidence suggests that Cx43 may be the predominant gap junction protein expressed in bone cells [11, 19, 29, 45, 47, 58]. However, Cx45 may be expressed by some osteoblastic cell lines [50]. Cell coupling in bone cells can be regulated by forskolin, cAMP [45] PTH [19, 45] retinoic acid, transforming growth factor ß [8] and pH [46, 57]. The regulation of cell coupling in bone by PTH is of particular interest since cell coupling also sensitizes osteoblastic cells to PTH [53]. Recent data also suggests the cell coupling contributes to the mechanism by which bone cells integrate and amplify biophysical signals. Considering the effect of PTH on coupling, gap junctional intercellular communication may be a cellular mechanism whereby hormonal signals act in synergy with biophysical factors to affect bone cell metabolism. Thus, PTH, by increasing coupling, could increase the sensitivity of osteoblastic networks to biophysical signals such as load-induced electric fields. This hypothesis, while attractive, awaits experimental verification.

Acknowledgements

References

1. Akisaka T, Yoshida H, Kogaya Y, Kim S, Yamamoto M, Kataoka K (1990) Membrane modifications in chick osteoclasts revealed by freeze-fracture replicas. Amer J Anat **188**, 381-392.

2. Bastiaanse EM, Jongsma HJ, van der Laarse A, and Takens-Kwak BR (1993) Heptanol-induced decrease in cardiac gap junctional conductance is mediated by a

decrease in the fluidity of membranous cholesterol-rich domains. J Membrane Biol **136**, 135-145.

3. Benayahu D, Fried A, Shamay A, Cunningham N, Blumberg S, Wientroub S (1994) Differential effects of retinoic acid and growth factors on osteoblastic markers and CD10/NEP activity in stromal-derived osteoblasts. J Cell Biochem **56**, 62-73.

4. Beyer EC (1993) Gap junctions. Int Rev Cytol **137**, 1-37.

5. Beyer EC, Kistler J, Paul DL, Goodenough DA (1989) Antisera directed against connexin43 peptides react with a 43-kD protein localized to gap junctions in myocardium and other tissues. J Cell Biol **108**, 595-605.

6. Beyer ED, Paul DL, Goodenough DA (1987) Connexin43: a protein from rat heart homologous to a gap junction protein from liver. J Cell Biol **105**, 2621-2629.

7. Boitano S, Dirksen ER, Sanderson MJ. (1992) Intercellular propagation of calcium waves mediated by inositol trisphosphate. Science **258**, 292-295.

8. Chiba H, Sawada N, Oyamada M, Kojima T, Iba K, Ishii S, Mori M (1994) Hormonal regulation of connexin 43 expression and gap junctional communication in human osteoblastic cells. Cell Struct Funct **19**, 173-177.

9. Chiba H, Sawada N, Oyamada M, Kojima T, Nomura S, Ishii S, Mori M (1993) Relationship between the expression of the gap junction protein and osteoblast phenotype in a human osteoblastic cell line during cell proliferation. Cell Struct Funct **18**, 419-426.

10. Christ GJ, Brink PR, Zhao W, Moss J, Gondre CM, Roy C, Spray DC (1993) Gap junctions modulate tissue contractility and alpha 1 adrenergic agonist efficacy in isolated rat aorta. J Pharmacol Exp Ther **266**, 1054-1065.

11. Civitelli R, Beyer EC, Warlow PM, Robertson AJ, Geist ST, Steinberg TH (1993) Connexin43 mediates direct intercellular communication in human osteoblastic cell networks. J Clin Invest **91**, 1888-1896.

12. Civitelli R, Fujimori A, Bernier SM, Warlow PM, Goltzman D, Hruska KA, Avioli LV. (1992) Heterogeneous intracellular free calcium responses to parathyroid hormone correlate with morphology and receptor distribution in osteogenic sarcoma cells. Endocrinology **130**, 2392-2400.

13. Cooper MS (1984) Gap junctions increase the sensitivity of tissue cells to exogenous electric fields. J Theor Biol **111**, 123-130.

14. Cooper MS, Miller JP, Fraser SE (1989) Electrophoretic repatterning of charged cytoplasmic molecules within tissues coupled by gap junctions by externally applied electric fields. Dev Biol **132**, 179-188.

15. Cowin SC, Weinbaum S, Zeng Y (1995) A case for bone canaliculi as the anatomical site of strain generated potentials. J Biomechanics **28**, 1281-1297.

16. Donahue HJ, Fryer MJ, Eriksen EF, Heath HD (1988) Differential effects of parathyroid hormone and its analogues on cytosolic calcium ion and cAMP levels in cultured rat osteoblast-like cells. J Biol Chem **263**, 13522-13527.

17. Donahue HJ, Guilak F, Van der Molen M, McLeod RJ, Rubin CT, Grande DA, Brink PR (1995) Chondrocytes isolated from mature articular cartilage retain the capacity to form functional gap junctions. J Bone Mineral Res **10**, 1359-1364.

18. Donahue HJ, Li Z, Zhou Z, Simon B (1995) Pulsed electromagnetic fields affect steady state levels of Cx43 mRNA in osteoblastic cells. J Bone Mineral Res **10**, S207.

19. Donahue HJ, McLeod KJ, Rubin CT, Andersen J, Grine EA, Hertzberg EL, Brink PR. (1995) Cell-to-cell communication in osteoblastic networks: cell line-dependent hormonal regulation of gap junction function. J Bone Mineral Res **10**, 881-889.

20. Doty SB (1981) Morphological evidence of gap junctions between bone cells. Calcif Tissue Int **33**, 509-512.

21. Duncan RL, Turner CH (1995) Mechanotransduction and the functional response of bone to mechanical strain [Review]. Calcif Tissue Int **57**, 344-358.

22. Fedotov VP, Gudoshnikov VI, Baranova IN (1993) [The possible participation of gap junctions in the realization of the effects of stimulators of secretory processes in the hypophysis]. Biul Eksper Biologii i Meditsiny **116**, 619-621.

23. Fukada E, Yasuda I (1957) On the piezoelectric effect of bone. J Phys Soc **2**, 1158-1162.

24. Gray C, Boyde A, Jones SJ (1996) Topographically induced bone formation in vitro: implications for bone implants and bone grafts. Bone **18**, 115-123.

25. Hansen M, Boitano S, Dirksen ER, Sanderson MJ (1995) A role for phospholipase C activity but not ryanodine receptors in the initiation and propagation of intercellular calcium waves. J Cell Sci **108**, 2583-2590.

26. Harris SA, Enger RJ, Riggs BL, Spelsberg TC. (1995) Development and characterization of a conditionally immortalized human fetal osteoblastic cell line. J Bone Mineral Res **10**, 178-186.

27. Hastings GW, Mahmud FA (1988) Electrical effects in bone. J Biomed Eng **10**, 515-521.

28. Jeansonne BG, Feagin FF, McMinn RW, Shoemaker RL, Rehm WS (1979) Cell-to-cell communication of osteoblasts. J Dental Res **58**, 1415-1423.

29. Jones SJ, Gray C, Sakamaki H, Arora M, Boyde A, Gourdie R, Green C (1993) The incidence and

size of gap junctions between the bone cells in rat calvaria. Anatomy Embryol **187**, 343-352.

30. Jongen JWJM, Willemstein-Van Hove C, Van Der Meer JM, Bos MP, Juppner H, Segre GV, Abou-Samra AB, Feyen JHM, Herrmann-Erlee MPM (1995) Down-regulation of the receptor for parathyroid hormone (PTH) and PTH-related peptide by transforming growth factor-B in primary fetal rat osteoblasts. Endocrinology **136**, 3260-3266.

31. Lanyon LE (1993) Osteocytes, strain detection, bone modeling and remodeling. Calcif Tissue Int **53**, S102-S107.

32. Marino AA, Becker RO, Soderholm SC (1971) Origin of the piezoelectric effect in bone. Calcif Tissue Res **8**, 177-180.

33. Martinez Arias A (1994) Pathways of cell communication during development: signalling and epistases. Trends in Genetics **10**, 219-222.

34. Mason DJ, Hillam RA, Skerry TM (1996) Constitutive in vivo mRNA expression by osteocytes of B-actin, osteocalcin, connexin-43, IGF-I, c-fos and c-jun, but not TNF-a nor tartrate-resistant acid phosphatase. J Bone Mineral Res **11**, 350-357.

35. McLeod KJ (1992) Microelectrode measurements of low frequency electric field effects in cells and tissues. [Review]. Bioelectromagnetics **Suppl 1**, 161-178.

36. McLeod KJ, Donahue HJ, Levin PE, Fontaine MA, Rubin CT (1993) Electric fields modulate bone cell function in a density-dependent manner. J Bone Mineral Res **8**, 977-984.

37. Munari-Silem Y, Lebrethon MC, Morand I, Rousset B, Saez JM (1995) Gap junction-mediated cell-to-cell communication in bovine and human adrenal cells. A process whereby cells increase their responsiveness to physiological corticotropin concentrations. J Clin Invest **95**, 1429-1439.

38. Palumbo C, Palazzini S, Marotti G (1990) Morphological study of intercellular junctions during osteocyte differentiation. Bone **11**, 401-406.

39. Paul DL (1986) Molecular cloning of cDNA for rat liver gap junction protein. J Cell Biol **103**, 123-134.

40. Pilla AA, Nasser PR, Kaufman JJ (1992) Cell-cell communication significantly decreases thermal noise limits for electromagnetic bioeffects. Proc 14th Ann Intern Conf IEEE Eng in Med & Biol Soc (Part 1) **7**, 300.

41. Rubin CT, McLeod KJ, Lanyon LE (1989) Prevention of osteoporosis by pulsed electromagnetic fields. J Bone Joint Surgery - Amer Vol **71**, 411-417.

42. Saez JC, Berthoud VM, Moreno AP, Spray DC (1993) Gap junctions. Multiplicity of controls in differentiated and undifferentiated cells and possible functional implications. Adv Second Messenger

Phosphoprotein Res **27**, 163-198.

43. Sanderson MJ, Charles AC, Boitano S, Dirksen ER (1994) Mechanisms and function of intercellular calcium signaling. Mol Cell Endocrinol **98**, 173-187.

44. Sanderson MJ, Charles AC, Dirksen ER (1990) Mechanical stimulation and intercellular communication increases intracellular Ca^{2+} in epithelial cells. Cell Regulation **1**, 585-596.

45. Schiller PC, Mehta PP, Roos BA, Howard GA (1992) Hormonal regulation of intercellular communication: parathyroid hormone increases connexin 43 gene expression and gap-junctional communication in osteoblastic cells. Mol Endocrinol **6**, 1433-1440.

46. Schirrmacher K, Brummer F, Dusing R, Bingmann D (1993) Dye and electric coupling between osteoblast-like cells in culture. Calcif Tissue Int **53**, 53-60.

47. Schirrmacher K, Schmitz I, Winterhager E, Traub O, Brummer F, Jones D, Bingmann D (1992) Characterization of gap junctions between osteoblast-like cells in culture. Calcif Tissue Int **51**, 285-290.

48. Shapiro F (1988) Cortical bone repair. J Bone Joint Surgery **70A**, 1067-1081.

49. Stauffer PL, Zhao H, Luby-Phelps K, Moss RL, Star RA, Muallem S (1993) Gap junction communication modulates $[Ca^{2+}]i$ oscillations and enzyme secretion in pancreatic acini. J Biol Chem **268**, 19769-19775.

50. Steinberg TH, Civitelli R, Geist ST, Robertson AJ, Hick E, Veenstra RD, Wang HZ, Warlow PM, Westphale EM, Laing JG, et al. (1994) Connexin43 and connexin45 form gap junctions with different molecular permeabilities in osteoblastic cells. EMBO Journal **13**, 744-750.

51. Turner CH (1992) Functional determinants of bone structure: beyond Wolff's law of bone transformation [editorial]. Bone **13**, 403-9.

52. Vander Molen MA, McLeod KJ, Donahue HJ, Rubin CT (1996) The influence of intercellular communication in defining osteoblast activity to biophysical stimuli. Trans Ortho Res Soc **2**, 338.

53. Vander Molen MA, Rubin CT, McLeod KJ, McCauley LK, Donahue HJ. (1996) Gap junctional intercellular communication contributes to hormonal responsiveness in osteoblastic networks. J Biol Chem **271**, 12165-12171.

54. Weaver JC, Astumian RD (1990) The response of living cells to very weak electric fields: the thermal noise limit [published erratum appears in Science 1990 March 2; **247**(4946):1019]. Science **247**, 459-462.

55. Wolburg H, Rohlmann A (1995) Structure-function relationships in gap junctions. Int Rev Cytol **157**, 315-356.

56. Xia SL, Ferrier J (1992) Propagation of a

calcium pulse between osteoblastic cells. Biochem Biophys Res Commun **186**, 1212-1219.

57. Yamaguchi DT, Huang JT, Ma D (1995) Regulation of gap junction intercellular communication by pH in MC3T3-E1 osteoblastic cells. J Bone Mineral Res **10**, 1891-1899.

58. Yamaguchi DT, Ma D, Lee A, Huang J, Gruber HE (1994) Isolation and characterization of gap junctions in the osteoblastic MC3T3-E1 cell line. J Bone Mineral Res **9**, 791-803.

59. Yancey SB, Biswal S, Revel JP (1992) Spatial and temporal patterns of distribution of the gap junction protein connexin43 during mouse gastrulation and organogenesis. Development **114**, 203-212.

60. Zhang JT, Nicholson BJ (1989) Sequence and tissue distribution of a second protein of hepatic gap junctions, Cx26, as deduced from its cDNA. J Cell Biol **109**, 3391-3401.

Discussion with Reviewers

R.L. Duncan: The authors state that they and other laboratories have demonstrated that Lucifer yellow can permeate gap junctions and that gap junctions are necessary for the propagation of calcium waves in osteoblast monolayers. However, it is not clear what molecule is communicated to the surrounding cells. Boitano *et al.* [7] have demonstrated that calcium waves in lung epithelia are induced by the movement of IP$_3$ through gap junctions to initiate intracellular calcium release. Has any work been done to determine the permeant molecule of Cx43 and could a similar mechanism to the one observed in lung epithelia be at work in osteoblasts?

Authors: While Sanderson, Boitano and colleagues [7, 43, 44] have demonstrated that IP$_3$ propagates the mechanically-induced Ca^{2+} signal in epithelial cells, it is not clear that this is the case in bone cells. Whereas in epithelial cells the Ca^{2+} signal propagates in the absence of extracellular Ca^{2+}, in osteoblasts the Ca^{2+} signal does not propagate in the absence of extracellular Ca^{2+}, suggesting a different mechanism than that which operates in epithelial cells.

R.L. Duncan: Have you examined any possible gating mechanisms of gap junctions in osteoblasts, in particular, the role of the cytoskeleton or membrane potential?

Authors: We have as yet not examined the role of the cytoskeleton or membrane potential in gap junction gating in osteoblasts.

R.L. Duncan: The authors demonstrate that ROS 17/2.8 cells transfected with antisense deoxynucleotides have a reduced cyclic AMP response to PTH when compared to normal control ROS cells. With the knowledge that long chain alcohols may produce deleterious effects on cell function, do these agents produce a similar reduction in PTH-induced cAMP accumulation in the normal osteoblast as in the antisense transfected osteoblast?

Authors: The purpose of developing Cx43 antisense transfected clones was to avoid the deleterious effects of long chain alcohols. Therefore, we have not examined the effect of these agents on PTH-stimulated cAMP accumulation. However, we have demonstrated that subconfluent ROS 17/2.8 cells display reduced coupling and reduced responsiveness to PTH, consistent with a role of cell-to-cell communication in hormonal responsiveness.

R.L. Duncan: Osteoblasts proceed through a defined differentiation pattern resulting in mineralization of the bone matrix and culminating in a relatively quiescent phenotype termed lining cells. Has anyone examined the role of gap junctional communication on differentiation of the osteoblast, i.e., the effects of gap junctional blockade on differentiation markers such as type-I collagen?

Authors: This is a very interesting question which we are currently pursuing. Civitelli *et al.* [62] have demonstrated that both non-differentiated and well differentiated osteoblasts express abundant Cx43 and are well coupled. Furthermore, dexamethasone which stimulates differentiation does not stimulate dye coupling or Cx43 expression. Thus, it was suggested that the presence of functionally active gap junctions is not dependent on any particular phase of osteoblast differentiation. On the other hand, we have unpublished data which suggest that in hFOB cells Cx43 expression parallels the expression of alkaline phosphatase, osteocalcin and mineralization as a function of time in culture. Additionally, growing these cells at 39.5°C which stimulates differentiation also stimulates Cx43 expression. Clearly more research is needed to elucidate the role of gap junctions in osteoblastic differentiation.

R.L. Duncan: The authors have observed that electro-magnetic fields decrease the mRNA of Cx43 as well as protein expression in ROS 17/2.8 cells. How long must the field be in place before a reduction in expression and production of Cx43 is observed? What is the duration of the inhibition of the expression of Cx43 following exposure to electromagnetic fields?

Authors: Our preliminary data suggest that a 72 hour exposure to pulsed electromagnetic fields decreases Cx43 expression and dye transfer. We have not yet examined the duration of this inhibition.

J. Bidwell: The authors demonstrate that the cAMP response to PTH was reduced in Cx43-deficient clones relative to normal ROS 17/2.8 cells and control transfectants (Fig. 2). The authors go on to speculate that coupling of osteoblasts by gap junctions may act to amplify the effect of receptor activation of a single cell by permitting the spread of second messenger to adjacent cells that are not directly activated by PTH. However, the "spread of second messengers" through a bone cell network would dilute cAMP levels in the primary and secondary target cells and would not result in a net increase in this second messenger in the network (introducing cAMP into a cell through a gap junction should not induce the production of more cAMP in this same cell). In fact one would predict that such a scheme would attenuate the cellular response to cAMP at the level of gene expression. (A) How would the authors respond to this criticism? (B) Would paracrine interactions serve to amplify a primary signal more effectively than diffusion of second messengers? (C) Do the authors have any evidence that the response to PTH at the level of transcription (e.g., nuclear run-off experiments) is modulated in Cx43-deficient cells?

Authors: (A) The concept that cAMP spread from cell-to-cell would dilute the cAMP levels makes sense if cAMP is the signal passing from cell-to-cell. However, the observation that PTH-stimulated cAMP accumulation is potentiated in highly coupled cells suggests that cAMP is not passing from cell-to-cell. The more likely candidates are IP_3 or cytosolic Ca^{2+}.

(B). While it is very likely that cell-to-cell paracrine signaling occurs it is unlikely that it would be more efficient. First, under most conditions any paracrine signal used for intercellular signaling would be subject to dilution within the extracellular matrix. Secondly, the directionality of paracrine signals would presumably be more difficult to control than signaling through gap junctions. Finally, gap junctions are conducting electrical signals which would of course travel much faster than any paracrine signal diffusing throughout the extracellular matrix.

(C). We do not have any data on PTH response at the level of gene transcription in coupling-deficient cells. We are very interested in examining this important issue.

J. Bidwell: It has been suggested [61] that the coupling of osteocytes, lining cells and osteoblasts by gap junctions may form a "bone membrane" and that osteocytes may act as mechanosensory cells and through gap junctions, transmit mechanically-induced signals (cAMP, ions, or prostaglandins) to osteoblasts ultimately for the regulation of osteoclast activity. This proposed directed signal transmission from one osteocyte to one osteoblast seems less problematic than the diffusion of a second messenger signal throughout a bone cell network as proposed by the authors. Please comment.

Authors: It is unclear how transmission of a signal from osteocytes to osteoblasts would be less problematic than between osteoblasts and osteoblasts. Indeed, it is possible that both routes employ similar transmission mechanism. In either case, gap junctional communication could function to integrate and amplify mechanical signals.

J. Bidwell: Could gap junctions serve the purpose of maintaining tissue integrity, i.e., ensuring that the mature osteoblasts maintain the row of cells organization observed along the matrix surface as opposed to a signalling function?

Authors: This is certainly a possibility and the two functions are not mutually exclusive.

Additional References

61. Aarden EM, Burger EH, Nisweide PJ (1994) Function of osteocytes in bone. J Cell Biochem **55**, 287-299.

62. Civitelli R (1993) Cell-cell communication through gap junctions in bone: relevance to bone cell physiology. Ital J Mineral Eiectrolyte Metab 7:165-174.