Gap Junctional Intercellular Communication Contributes to Hormonal Responsiveness in Osteoblastic Networks

Monique A. Vander Molen, Clinton T. Rubin, Kenneth J. McLeod, Laurie K. McCauley, and Henry J. Donahue

From the iMusculo-Skeletal Research Laboratory, Department of Orthopaedics, State University of New York, Stony Brook, New York 11794-8181, the iUniversity of Michigan, School of Dentistry, Ann Arbor, Michigan 48109-1078, and the Musculoskeletal Research Laboratory, Departments of Orthopaedics and Rehabilitation and Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033-0850

To evaluate whether intercellular coupling via connexin43 gap junction channels modulates hormonal responsiveness of cells in contact, we have created osteoblastic cell lines deficient in connexin43. Osteoblastic ROS 17/2.8 cells were transfected with a plasmid containing an antisense cDNA construct to rat connexin43. Control transfection did not alter cell-to-cell coupling nor connexin43 mRNA or protein expression relative to nontransfected ROS 17/2.8 cells. In contrast, stable transfection with an antisense connexin43 cDNA resulted in two clones, RCx4 and RCx16, which displayed significant decreases in connexin43 mRNA and protein expression and were dramatically deficient in cell-to-cell coupling. Phenotypically, all transfectants retained osteoblastic characteristics. However, cells rendered connexin43-deficient through antisense transfection displayed a dramatic attenuation in the cAMP response to parathyroid hormone. Alterations in hormonal responses were not due to changes in parathyroid hormone receptor number or binding kinetics nor to alterations in adenyl cyclase activity. These results indicate that gap junctions may be required for mediating hormonal signals. Furthermore, these experiments support a regulatory role for connexin43-mediated intercellular communication in the modulation of hormonal responses within elaborately networked bone cells.

Although evidence of a critical role for gap junctional intercellular communication in tissue development and morphogenesis is emerging (1–4), the role of cell-to-cell coupling in fully developed tissue is less clear. Recent investigations, however, suggest that gap junctional coupling contributes to the coordinated responses of cellular networks to extracellular signals. For instance, pharmacological inhibitors of intercellular coupling have been shown to inhibit adenocorticotrophic hormone-induced steroidogenesis in adrenal cells (5), bombesin-stimulated cytosolic calcium oscillations in pancreatic acini (6), the secretory effect of thyrotrophic releasing hormone on pituitary cells (7), and alpha2 adrenergic receptor agonist-stimulated contractions in smooth muscle cells (8). However, these studies are difficult to interpret because the agents used to inhibit gap junctional coupling, in most cases lipophilic long chain alcohols, may have many nonspecific effects on cells (9). More precise approaches, which directly and specifically inhibit intercellular coupling in a particular cell type, are required to better understand gap junctional influences on cellular responses to extracellular signals.

To more directly and specifically assess the role of intercellular communication on the ability of cell ensembles to respond to extracellular signals, we developed two clonal osteoblastic cell lines with markedly reduced dye coupling and expression of connexin43 (Cx43).1 There are several reasons why osteoblastic cells present a useful paradigm to examine the role of intercellular communication in cellular responses to extracellular signals. Osteoblasts communicate with one another via gap junctions both in organ culture (10) and in vitro (11–16), and cell-to-cell communication is believed to be critical for the coordinated cell behavior necessary in bone tissue development (17–20). Additionally, the particular gap junction proteins expressed by osteoblasts have been characterized (11–16), and osteotropic hormones, such as PTH, have been shown to regulate cell coupling in osteoblastic cells (16, 21, 22). Finally, there is considerable evidence for a cell density-dependent and possibly intercellular coupling-dependent effect of extracellular signals on osteoblastic cell networks (23–25). Therefore, to examine the hypothesis that cell-to-cell coupling is critical for the coordinated response of cellular networks to extracellular signals, we rendered an osteoblastic cell line, ROS, Cx43-deficient. ROS were chosen because they express phenotypic characteristics typical of well differentiated osteoblasts (26, 27) and demonstrate abundant gap junctions (14, 16).

Cx43 deficiency was accomplished through stable transfection of a plasmid DNA containing cDNA antisense to the mRNA of rat Cx43, the predominant connexin isoform in bone (11–16, 28). Antisense transfectants were found to be functionally uncoupled relative to controls and maintained their osteoblastic phenotype. However, cells deficient in functional coupling displayed a dramatic decrease in response to PTH.

EXPERIMENTAL PROCEDURES

Materials—Forskolin and dbcAMP were obtained from Calbiochem. PTHrP(1–36), rPTH(1–34), and [Tyr36]PTHRP(1–36) were obtained from Bachem. Cyclic AMP-125I radioimmunoassay kits were purchased from DuPont NEN. Deoxyadenosine 5-triphosphate ([α-32P]dCTP) (3000 Ci/mmol) and [125I]Na were obtained from ICN Biomedicals. Random primed DNA labeling kits were obtained from Boehringer Mannheim. Plasmids for transfection and the cDNA probe for rat Cx43 were obtained from Boehringers Mannheim.

The abbreviations used are: Cx43, connexin43; PTH, parathyroid hormone; ROS, rat osteosarcoma 17/2.8 cells; dbcAMP, dibutyryl cAMP; rPTH(1–34), rat PTH fragment 1–34; PTHrP, PTH-related protein; PTHR(1–36), human recombinant PTH-related protein fragment 1–36; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kb, kilobase(s); PB, 0.1 M phosphate buffer.

*This work was supported by National Institutes of Health Grants AG13087 (to H. J. D.), AG10199 (to H. J. D.), ES06287 (to K. J. M.), and the Musculoskeletal Research Laboratory, Departments of Orthopaedics and Rehabilitation and Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033-0850.

1 The abbreviations used are: Cx43, connexin43; PTH, parathyroid hormone; ROS, rat osteosarcoma 17/2.8 cells; dbcAMP, dibutyryl cAMP; rPTH(1–34), rat PTH fragment 1–34; PTHrP, PTH-related protein; PTHR(1–36), human recombinant PTH-related protein fragment 1–36; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kb, kilobase(s); PB, 0.1 M phosphate buffer.

To whom correspondence should be addressed. Tel.: 717-531-4819; Fax: 717-531-7583.
from Glenn I. Fishman (Albert Einstein College of Medicine, Bronx, NY). Affinity-purified antipeptide antibody to rat Cx43 (as described in Ref. 30) was provided by Elliot L. Hertzberg (Albert Einstein College of Medicine, Bronx, NY). The DNA probe for osteocalcin was from Gideon A. Rodan (Merk sharper and Dohme Research Laboratories, West Point, PA). The chicken type I collagen cDNA probe was provided by Paul S. Grinnell (University of New York, NY). The GAPDH cDNA probe was prepared by Philippe Fort (University des Sciences et Techniques du Languedoc, Montpellier Cedex, France). The DNA probe for PTH/PTHrP receptor was provided by Abdul-Badi Abou-Samra (Harvard Medical School, Boston, MA). Tissue culture medium, Geneticin, and Lipofectamine (Life Technologies, Inc.), tissue culture flasks (Falcon), fetal bovine serum (Hydne Laboratories), electrophoresis reagents, and nitrocellulose (Bio-Rad), X-OMAT AR film (Kodak), and nylon membranes (Schleicher & Schuell) were obtained from the designated sources. 6-Carboxyfluorescein (376 Da) was obtained from Molecular Probes, and microelectrode tips were from World Scientific. Horseradish peroxidase-conjugated anti-rabbit IgG, fluorescein-conjugated anti-rabbit IgG and ECL Western blotting detection reagents were obtained from Amersham Corp.. All other reagents and supplies including the alkaline phosphatase diagnostic kit were from Sigma.

Cell Culture Antisense Transfection, and Quantification of Dye Coupling—ROS were provided by Janet Rubin (Emory University, Atlanta, GA) and cultured as described previously (16). For transfection, ROS were plated at 5 × 10⁴ cells/cm² on 100-mm dishes and grown for 36 h. Cells were transfected with a pRC plasmid (Invitrogen) driven by the cytomegalovirus promoter containing an antisense oriented 248-base pair fragment corresponding to the first 82 amino acids of rat Cx43. Control cells (bG) were transfected with a similar plasmid containing a fusion of the bacterial β-galactosidase and neomycin genes. Cells were incubated for 6 h in 2 μg of plasmid DNA that had been incorporated into 70 μg of Lipofectamin liposomes per plate in serum-free OPTI-MEM medium. Cells were then refed with serum containing Ham's F-12 (1:1) medium and maintained in culture for 48 h before antibiotic selection in Geneticin (G418)-containing medium (400 μg/ml). After multiple rounds of selection, antisense-transfected clones (RCx) were chosen for their inability to transfer dye to neighboring cells. Dye quantitation was as described in Donahue et al. (16). For some experiments a vehicle control (ethanol) or dbAMP at a final concentration of 10 μM was added prior to initial dye loading.

RNA Isolation and Northern Blot Analysis—Total RNA was extracted by previously published methods (31) from cells on 100-mm dishes. Total RNA (20 μg) was denatured, separated, and transferred to a nylon solid support. Blots were hybridized with 2 × 10⁶cpm/1×10⁹ probe of an 1.8-kb BamHI-NcoI fragment of the rat PTHrP receptor cDNA (32), a 0.89-kb Avai fragment of human heart Cx43 cDNA (29), a 0.52-kb EcoRI fragment of rat osteocalcin cDNA (33), an 1.8-kb PstI-HindIII fragment of the chicken type I collagen cDNA (34), or an 1.4-kb PstI fragment of rat GAPDH cDNA (35). Hybridizations and washes were performed according to the manufacturer's instructions (Schleicher & Schuell), followed by exposure of high pressure liquid chromatography-purified, monodinated [32P]PTHrP(1-36) in addition to varying concentrations of nonradioactive PTHrP(1-36). Cells were incubated at 4°C for 2 h, followed by removal of unbound peptide, cell lysis, and determination of bound radioactivity by scintillation counting. Parallel cultures were enumerated by trypan blue dye exclusion. The numbers of receptors per cell were calculated from the Bmax values obtained through Scatchard transformation.

Statistical Analysis—All data are reported as means ± S.E. except as noted. One-way analysis of variance followed by Student Neuman Kuells test were used to analyze cell coupling data and cAMP accumulation under various conditions; p values < 0.05 were considered significant.

RESULTS

Cx43 Antisense Transfectants Are Communication-deficient—Transfection with an antisense Cx43 construct produced two doses, RCx4 and RCx16, which consistently exhibited a reduced capacity to transfer dye. Fig. 1 illustrates a typical experiment in which ROS (top) display abundant dye coupling, whereas antisense transfectant, RCx16 (bottom), do not. Fig. 2 summarizes the degree of coupling in ROS, control transfecntants (bG), RCx16, and RCx4. ROS and bG displayed similar levels of dye coupling after 5 min (15.5 ± 2.1 and 13.9 ± 1.9, mean ± S.E. number of neighboring cells with dye, respective...
ly). At no time did either ROS or bG, when dye loaded, communicate with less than 10 neighboring cells. However, in the antisense transfectants, RCx4 and RCx16, coupling was reduced more than 9-fold (1.2 ± 1.1 and 1.5 ± 0.8, mean ± S.E. number of neighboring cells with dye, respectively). Thus, although dye coupling was not eliminated, it was dramatically reduced. This reduction was maintained in cells even 30 min after dye loading.

Gap junction deficiency in both RCx4 and RCx16 was verified by the addition of dbcAMP at 10 μM, a concentration found to enhance functional coupling by as much as 50% in ROS (Fig. 3). These results demonstrate dbcAMP increased coupling in both ROS and bG by 49.8 and 48.6%, respectively. Functional coupling increased within 10 min in both ROS and bG and peaked in ROS by 30 min and in bG by 40 min. On the other hand, dye coupling was unaffected by dbcAMP in RCx16 and RCx4 (6.7 and 0.92% increase over basal, respectively).

To determine if uncoupling with antisense transfection was reflected in Cx43 mRNA levels, Northern blot analysis was performed on transfected and nontransfected ROS. Fig. 4A demonstrates that both ROS (lane 1) and control transfectants, bG (lane 2), expressed abundant Cx43 mRNA of approximately 3.0 kb in size. Cx43-deficient clones, RCx4 (lane 3) and RCx16 (lane 4), displayed virtual elimination of Cx43 mRNA expression. Normalization of mRNA abundance to GAPDH, a gene with transcription levels in ROS that remained stable independent of plating density, revealed that mRNA levels in RCx16 and RCx4 were reduced by 78 and 92%, respectively, relative to ROS (Fig. 4B).

Western blot analysis of transfected and nontransfected cells revealed a pattern of Cx43 protein expression similar to that observed for mRNA expression and functional coupling experiments (Fig. 5). ROS (lane 1) and bG (lane 2) showed the predominant presence of a 43-kDa band consistent with the nonphosphorylated form of Cx43 (Cx43-NP) from rat heart (3). ROS and bG also expressed two bands (Cx43-P1 and Cx43-P2) at reduced levels relative to Cx43-NP, also consistent with the expression of two phosphorylated forms of Cx43 (3). In both antisense transfected clones, RCx4 and RCx16, a greater than 86% reduction in expression for all forms of Cx43 protein was observed using the polyclonal antibody to rat Cx43.

Indirect immunostaining of ROS (Fig. 6A) revealed punctate distribution of Cx43 immunoreactivity particularly abundant at the interface of adjoining cells. Numerous immunoreactive sites within the cytoplasm, located primarily along the perinuclear border, were also apparent. Although this pattern of staining was observed in bG, there was no Cx43 immunoreactivity at the cell membrane interface or within the cytoplasm in either RCx4 (data not shown) or RCx16 (Fig. 6B).

Transfected Cells Maintain Osteoblastic Phenotypic Characteristics—Preliminary results demonstrated that transfected cells proliferated at different rates in the presence of G418-containing medium. For this reason, all experiments, including dye loading and phenotyping, were performed in medium lacking G418. However, neither the expression of Cx43 nor the...
coupling capacity in antisense transfectants were affected by the lack of G418 for up to 3 weeks.

Osteoblastic characteristics include expression of type I collagen, osteocalcin, and PTH/PTHrP receptor mRNA; therefore, Northern blot analysis was performed to identify any loss of these osteoblastic phenotypic markers associated with transfection. RCx4, RCx16, and bG as well as ROS demonstrated a uniform expression for the 3.0-kb type I collagen transcript, the 0.6-kb osteocalcin transcript, and the 2.3-kb PTH/PTHrP receptor transcript as assessed by quantification of total cellular RNA normalized to GAPDH (data not shown). Another characteristic of the osteoblast phenotype is the presence of alkaline phosphatase activity, a marker for osteoblastic differentiation that is associated with mineralization (42). We found alkaline phosphatase abundance was similar in ROS, bG, RCx4, and RCx16 (data not shown).

Attenuated Hormonal Responsiveness in Cx43-deficient Osteoblastic Cells—To examine the functional importance of intercellular coupling, PTH-stimulated cAMP accumulation was examined in well-coupled ROS and bG and in poorly coupled Cx43-deficient cells. In ROS and bG, PTH(1–34) stimulated cAMP accumulation in a concentration-dependent manner (Fig. 7A). Significant cAMP accumulation was detected at $10^{-11}$ M PTH, a physiological concentration, in both ROS and bG, and maximal (13-fold) stimulation occurred at $10^{-7}$ M PTH. On the other hand, PTH-stimulated cAMP accumulation was dramatically attenuated, relative to ROS, in Cx43-deficient RCx16 and RCx4. In RCx16 and RCx4, the maximal cAMP response to PTH occurred at $10^{-7}$ and $10^{-8}$ M, respectively, and was only 26.6 and 21.9%, respectively, of the maximal response in ROS. Estimated $E_{50}$ values for each cell line were determined from dose-response curves for each experiment (ROS, $1.90 \times 10^{-11}$ M; bG, $1.74 \times 10^{-11}$ M; RCx4, $2.15 \times 10^{-11}$ M; RCx16, $1.99 \times 10^{-11}$ M; n = 3–6).

To determine whether decreased hormonal responsiveness in antisense transfectants could be due to reductions in PTH binding or receptor availability, radiolabeled binding studies were performed on cells at equivalent densities. Radiolabeled parathyroid hormone-related protein, PTHrP(1–36), a ligand with binding kinetics for the PTH/PTHrP receptor equivalent to PTH (41), was found to specifically bind to the receptor protein in all cells analyzed (Table I). These data confirm that bG, RCx4, and RCx16 contain a receptor protein that binds PTH analogs. From nonlinear regression of competition binding curves for PTHrP, the $E_{50}$ and the number of specific binding sites were obtained. Both bG and RCx16 bound PTHrP with affinities equivalent to ROS, whereas RCx4 was found to exhibit an increased affinity for the receptor. Scatchard transformation, used to estimate receptor densities from cells in late log phase growth, revealed that ROS, bG, and RCx16 expressed similar numbers of PTH/PTHrP surface receptors per cell. Cx43-deficient RCx4, with a 2.5-fold higher receptor binding affinity, were found to express fewer PTH/PTHrP receptors per cell. To determine whether the adenylyl cyclase in transfected cells was fully functional and to verify a uniform response of the enzyme in the absence of intercellular connectivity, cells were exposed to $10^{-11}$, $10^{-9}$, and $10^{-7}$ M forskolin, a direct activator of adenylyl cyclase. Forskolin-stimulated cAMP accumulation was similar in all cells examined (Fig. 8).

We also examined hormonal responsiveness in subconfluent ROS and bG. Subconfluent cells displayed less cell-to-cell contact and, consequently, up to 80% reduction in cell coupling (Fig. 2). Additionally, PTH-stimulated cAMP accumulation in subconfluent cells was significantly (p < 0.05) reduced relative to confluent cells (Fig. 7B). In subconfluent ROS and bG, exposure to $10^{-7}$ M PTH resulted in an approximately 6-fold increase in cAMP over basal, whereas in confluent cells this exposure resulted in a 13-fold increase in cAMP accumulation.
In osteoblastic cells such as ROS, PTH, via binding to its receptor, activates multiple second messenger systems, including the cAMP-protein kinase A cascade (43). Indeed, we found that PTH-stimulated cAMP accumulation in a concentration-dependent manner in both ROS (thick solid line) and bG (thick dashed line). However, PTH-stimulated cAMP accumulation in RCx4 (thin solid line) and RCx16 (thin dashed line) was dramatically attenuated. The peak cAMP response to $10^{-7}$ mPTH(1–34) in RCx4 and RCx16 was only 26.2 and 21.9% of that of ROS, respectively. In B, the intracellular cAMP response of ROS (thin solid line) and bG (thin dashed line) plated at 5–10,000 cells/cm² and grown for 48 h are shown. An 80% reduction in functional coupling in these low density cultures resulted in the attenuation of PTH-stimulated cAMP by 50 and 55% in ROS and bG, respectively. Confluent cultures of ROS (thick solid line) and bG (thick dashed line) are shown for comparison and are the same values as those presented in A. The average basal cAMP response from all experiments was 81 ± 17 pmd/million cells. All data represent means ± S.E. of three to six experiments performed in duplicate. *, significantly different from either ROS or bG ($p < 0.05$).

The diminished intracellular cAMP values found in subconfluent ROS and bG, cells that were poorly coupled, were similar to those exhibited by Cx43-deficient antisense transfectants.

### Discussion

In order to define a physiological function for cell-to-cell communication in ensembles of bone cells, we examined hormonal responsiveness in uncoupled transfectants that maintained phenotypic characteristics typical of osteoblastic cells. PTH was chosen because it is a potent regulator of bone metabolism exerting a direct effect on osteoblastic behavior (42). In osteoblastic cells such as ROS, PTH, via binding to its receptor, activates multiple second messenger systems, including the CAMP-protein kinase A cascade (43). Indeed, we found that PTH stimulated cAMP accumulation in a concentration-dependent manner in ROS, as has been previously demonstrated (39, 41). However, in antisense transfectants RCx4 and RCx16, cells exhibiting decreased Cx43 and, consequently, decreased coupling, PTH-stimulated cAMP accumulation was dramatically attenuated. Additionally, we found that subconfluent ROS and bG, cells that display decreased coupling, similarly demonstrated decreased cAMP responses to PTH. However, the cAMP response to PTH was greater in subconfluent ROS and bG than in antisense transfectants, probably because coupling was greater in subconfluent ROS and bG relative to antisense transfectants. Taken together, these data indicate that a decrease in cell-to-cell coupling contributes to decreased hormonal responsiveness in ROS.

A possible explanation for this decreased hormonal responsiveness in RCx4 and RCx16 could be an alteration in PTH/PTHrP receptor gene expression, protein abundance, or binding kinetics possibly as a result of plasmid transfection per se. However, we found PTH/PTHrP receptor gene expression remained similar in all cell lines examined. Although radio-ligand binding studies revealed that one Cx43-deficient clone, RCx4, expressed fewer PTH/PTHrP receptors on the cell surface, RCx4 cells also demonstrated a higher (2.5-fold) binding affinity for the PTH analog. Thus, RCx4 cells may, by clonal selection, have been enriched for a higher affinity receptor, which could compensate for decreased receptor number. Furthermore, had the cells been adequately intercellularly connected, it is likely that the number of receptors present in RCx4 (approximately 11,300) would have been sufficient for maximal stimulation of cAMP by PTH (44–46). In any case, we also observed diminished PTH-stimulated cAMP accumulation in RCx16, another Cx43-deficient clone that did not demonstrate any reduction in the number of cell surface PTH/PTHrP receptors or alteration in receptor binding affinity. Thus, in these experiments, attenuation of PTH-stimulated cAMP accumulation in antisense transfectants cannot be explained by diminished PTH receptor expression, availability, or binding kinetics.

Another explanation for our results could be that antisense...
transfection resulted in a dysfunctional adenylyl cyclase or some other component of the CAMP generating mechanism. If this were the case, one would expect transfected cells to display an attenuated CAMP response to forskolin, a compound that directly activates the catalytic subunit of adenylyl cyclase. Yet, forskolin-stimulated CAMP accumulation was similar in ROS, bG, RCx4, and RCx16. This suggests that a defect in the mechanism by which CAMP is generated does not contribute to reduced PTH responsiveness in antisense transfecteds. However, associated with the activation of adenylyl cyclase is the translocation of at least one G-protein (32), and it is possible that transfection resulted in alterations in G-protein function. If increased G-protein coupled receptor binding energy (such as that observed with PTHrP binding in RCx4) were associated with a conformation that activated G-proteins, it would predict a lower ED₅₀ value for adenylyl cyclase response. Yet, we observed that upon direct activation of adenylyl cyclase, neither the ED₅₀ nor the maximal CAMP response differed between RCx4 and ROS, suggesting the transducing G-proteins were not disturbed with transfection or that the affinity of ligand binding to these receptors is dissociable from receptor activation properties.

Without any apparent defect in the generation of CAM in Cx43-deficient RCX4 and RCX16, our studies suggest that gap junctions may act as conduits for optimal intracellular responsiveness to hormones such as PTH. Perhaps, as suggested by Christ et al. (8), gap junctions act to amplify the effects of local receptor activation by permitting the spread of second messengers to adjacent cells that are not directly activated by the agonist. Thus, a mechanism may exist whereby agonist-stimulated increases in a second messenger, such as cytosolic Ca²⁺ in responsive cells could be communicated via gap junctions to nonresponsive cells wherein the activity of other second messenger systems, such as adenylyl cyclase, are potentiated (43, 47–49). More importantly, such a mechanism may not be unique to bone cells (5–8, 50).

Amplification of local receptor activity would be particularly important when there are variabilities in hormone receptor expression and/or in gap junctions between interconnected cells. With such variabilities, gap junctional coupling would regulate hormone-stimulated cellular behavior to various degrees in different cellular systems. Munari-Silem and co-workers (5) found that the adrenocorticotropic hormone-stimulated CAMP response in uncoupled adrenal cells was not altered even at submaximal hormone concentrations. Our findings suggest an attenuated CAMP response to PTH in Cx43-deficient cells at all hormone concentrations examined. These differences could be explained by a uniform or homogeneous adrenocorticotropic hormone-stimulated CAMP response within individual bovine adrenal cells. In fact, Civitelli et al. (51) have shown that among individual osteoblasts, the response to PTH is quite heterogeneous such that only 30% of osteoblastic cells would respond to maximal doses of 10⁻⁷ M PTH on an individual basis. Hence, in a population of cells exhibiting a heterogeneous response to a hormone on an individual basis, gap junctions could provide a mechanism by which the net ensemble response would be greater than the sum of the individual responding cells.

This role of gap junctions and their regional specificity in more mature osteoblasts may explain certain abnormalities or loss of cellular signaling observed in the aging skeleton. Indeed, in preliminary experiments, we find functional coupling is significantly reduced in osteoblastic cells isolated from 28-month-old (aged) rats relative to those isolated from 4-month-old (young) rats. The decreased CAMP response to PTH we (52) and others (53–55) have reported in osteoblastic cells isolated from aged rats is strikingly similar to the decreased CAMP response to PTH observed in antisense transfected Cx43-deficient cells. If the preliminary results are confirmed, they could have important implications regarding the interplay between age-related changes in gap junctional coupling and intracellular signaling.

In summary, using an antisense transfection strategy, we have provided the first direct evidence that in ROS 17/2.8 cells, a well differentiated osteoblastic cell line, intercellular coupling is predominately mediated via gap junctions composed of Cx43. By the selective elimination of a specific connexin, we have been able to demonstrate that decreasing the abundance of a single gap junction protein can alter the responsiveness of a cellular ensemble to a hormonal signal. More importantly, our data reveal that cell-to-cell communication may be a critical component in the pathway by which more mature cellular networks coordinate their responsiveness to extracellular signals.

Acknowledgments—We thank the following people for invaluable contributions: Peter R. Brink, and Glenn J. Fishman for critical comments, advice, and discussions; Louise Paya, Amy J. Koh, and Christopher A. Beecher for technical assistance, and Debbie Firestone for generous support and help in preparation of the manuscript.

REFERENCES

1. Wolburg, H., and Rohlmann, A. (1995) Int. Rev. Cytol. 157, 315–373
2. Yanovsky, S. B., Blobel, S., and Revel, J. P. (1992) Development 114, 203–212
3. Saez, J., Berthoud, V. M., Moreno, A. P., and Spray, D. C. (1993) Adv. Second Messenger Phosphoprotein Res. 27, 163–197
4. Martineau, A. (1994) Trends Cell Biol. 10, 219–222
5. Munari-Silem, Y., Lebrethon, M. C., Morand, I., Rousset, B., and Saez, J. M. (1995) J. Clin. Invest. 95, 1429–1439
6. Saez, J. M., Zhao, H., Luby-Phelps, K., Sos, R. L., Star, R. A., and Maullien, S. (1993) J. Biol. Chem. 268, 19769–19775
7. Fedotov, V. P., Gudoshikov, V., and Baranova, I. N. (1995) Byull. Eksp. Biol. Med. 116, 619–621
8. Christ, G. J., Brink, P. R., Zhao, W., Moss, J., Gondé, C. M., Roy, C., and Spray, D. C. (1993) J. Pharmacol. Exp. Ther. 266, 1054–1065
9. Bastaarache, E. M., Jongima, H. J., van der Laarse, A., and takens-Kwak, B. R. (1993) J. Membr. Biol. 135, 135–145
10. Jeansonne, B. G., Feagin, F. F., McMinn, R. W., Shoemaker, R. L., and Rehm, W. (1979) J. Dent. Res. 58, 1415–1423
11. Schirrmacher, K., Schmitz, I., Winterhager, E., Traub, O., Brummer, F. J., Jones, D., and Bingmann, D. (1992) Calf. Tissue Int. 51, 285–290
12. Civitelli, R., Beyer, E. C., Warlow, P. M., Robertson, A. J., Geist, S. T., and Steinberg, T. H. (1993) J. Clin. Invest. 91, 1888–1896
13. Chiba, H., Sawada, N., Oya, M., Kalme, T., Iba, K., Ishii, S., and Mori, M. (1994) Cell Struct. Funct. 19, 173–177
14. Steinberg, T. H., Civitelli, R., Geist, S. T., Robertson, A. J., Hide, E., Veenstra, R. D., Wang, H. Z., Warlow, P. M., Westphol, E. M., Laing, J. G., and Beyer, E. C. (1994) EMBO J. 13, 744–750
15. Yamaguchi, D. T., Ma, D., Lee, A., Huang, J., and Gruber, H. E. (1994) J. Bone Miner. Res. 9, 791–803
16. Donahue, H. J., McLeod, K. J., Rubin, C. T., Anderson, J. A., Grine, A. E., Hertzberg, E. L., and Brink, P. R. (1995) J. Bone Miner. Res. 10, 881–889
17. Zimmermann, B. (1984) Anat. J. 138, 351–363
18. Coelho, C. N. D., and Kosher, R. A. (1991) J. Cell Biol. 117, 881–889
19. Dealy, C. N., Beyer, E. C., Warlow, P. M., Westphol, E. M., Laing, J. G., and Beyer, E. C. (1994) Mol. Endocrinol. 8, 1415–1423
20. Egan, J. J., Gronowicz, G., and Redan, G. A. (1991) J. Cell Biol. 1433–1340
21. van der Plas, A., and Niweijs, P. J. (1998) Bone NY 21, 107–111
22. Schiller, P. C., Mehta, P. P., Roos, B. A., and Howard, G. A. (1992) Mol. Endocrinol. 6, 1423–1440
23. Egg, J. J., Gronowicz, G., and Redan, G. A. (1991) J. Cell Biol. 45, 93–100
24. Broen, M. E., Arlot, M. E., and Reeve, J. (1993) Bone NY 14, 473–479
25. McLeod, K. J., Donahue, H. J., Fontaine, M. A., and Rubin, C. T. (1993) J. Bone Miner. Res. 8, 977–984
26. Majeska, R. J., Rodan, S. B., and Rodan, G. A. (1980) Endocrinology 107, 1494–1503
27. Majeska, R. J., Nair, B. C., and Rodan, G. A. (1985) Endocrinology 116, 170–179
28. Jones, S. J., Gray, C., Sakamaki, H., Arora, M., Boyde, A., Gourdie, R., and Green, C. (1993) Anat. Embryol. 1888–1896
29. Fishman, G. I., Spray, D. C., and Leinwand, L. A. (1990) J. Membr. Biol. 12–21
30. Yamamoto, T., Ochalski, A., Hertzberg, E. L., and Nagy, J. J. (1990) Brain Res. 508, 313–319
Gap Junctions Contribute to Hormonal Responsiveness

12171

31. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
32. Abou-Samra, A. B., Juppner, H., Force, T., Freeman, M. W., Kong, X. F., Schipani, E., Urena, P., Richards, J., Bonventre, J. V., Potts, J. T., Jr., Kronenberg, H. M., and Segre, G. V. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2732–2736
33. Weinreb, M., Shinar, D., and Rodan, G. A. (1990) J. Bone Miner. Res. 5, 831–837
34. Lehrrach, H., Frischau, A. M., Hanahan, D., Wozney, J., Fuller, F., Crkvenjakov, R., Boedtker, H., and Doly, P. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 5417–5421
35. Fort, P., Marty, L., Piechaczyk, M., El-Sabrouty, S., Dani, C., Jeanteur, P., and Blanchard, J. M. (1985) Nucleic Acids Res. 13, 1431–1442
36. Bradford, M. M. (1976) Anal Biochem. 72, 248–254
37. Laemmli, U. K. (1970) Nature 227, 680–685
38. Saez, J. C., Spray, D. C., and Hertzberg, E. L. (1990) In Vitro Toxicol. 3, 69–86
39. Donahue, H. J., Fryer, M. J., Eriksen, E. F., and Heath, H., III (1988) J. Biol. Chem. 263, 13522–13527
40. McCauley, L. K., Beecher, C. A., Melton, M. E., Werkmeister, J. R., Juppner, H., Abou-Samra, A. B., Segre, G. V., and Rosol, T. J. (1992) Mol. Cell. Endocrinol. 101, 331–336
41. Abou-Samra, A. B., Uneno, S., Juppner, H., Keutmann, H., Potts, J. T., Jr., Segre, G. V., and Nussbaum, S. R. (1989) Endocrinology 125, 2215–2217
42. Rodan, G. A., and Rodan, S. B. (1984) Advances in Bone and Mineral Research Annual II, 2nd Ed., pp. 240–245, W. B. Saunders, Philadelphia
43. Orloff, J. J., Wu, T. L., and Stewart, A. F. (1989) Endocrinol. Rev. 10, 476–495
44. Demay, M., Mitchell, J., and Goltzman, D. (1985) Am. J. Physiol. 249, E437–E446
45. Denzot, S., Grey, H. M., and Sette, A. (1990) Science 249, 1028–1030
46. Harding, C. V., and Unanue, E. R. (1990) Nature 346, 574–576
47. Xia, S.-L., and Ferrier, J. (1992) Biochem. Biophys. Res. Commun. 186, 1212–1219
48. Pedic, W. A., Kohler, G., and Barr, S. (1981) Calcif. Tissue Int. 33, 409–416
49. Bos, M. P., van Leeuwen, J. P., and Herrmann-Erlee, M. P. M. (1991) J. Cell. Physiol. 147, 87–92
50. Stelke, J. H. (1993) Nature 365, 314–320
51. Civitelli, R., Fujimori, A., Bernier, S. M., Warlow, P. M., Goltzman, D., Hruska, K. A., and Avioli, L. V. (1992) Endocrinology 130, 2392–2400
52. Donahue, H. J., and Rubin, C. T. (1992) Bone Miner. 17, Suppl. 1, 123
53. Egrie, D., Martin, D., Vienne, A., Neve, P., and Schoutens, A. (1992) Bone (NY) 13, 355–361
54. Wong, G. L., and Ng, M. C. (1992) J. Bone Miner. Res. 7, 701–708
55. Modrowski, D., and Marie, P. J. (1993) Cell Tissue Res. 271, 499–505