Integrin Dependence of Brain Natriuretic Peptide Gene Promoter Activation by Mechanical Strain*

Expression of the brain natriuretic peptide (BNP) gene in cultured neonatal rat ventricular myocytes is activated by mechanical strain in vitro. We explored the role of cell-matrix contacts in initiating the strain-dependent increment in human BNP (hBNP) promoter activity. Coating the culture surface with fibronectin effected a dose-dependent increase in basal hBNP luciferase activity and amplification of the response to strain. Preincubation of myocytes with an RGD peptide (GRGDS) or with soluble fibronectin, each of which would be predicted to compete for cell-matrix interactions, resulted in a dose-dependent reduction in strain-dependent hBNP promoter activity. A functionally inert RGE peptide (GRGES) was without effect. Using fluorescence-activated cell sorting, we demonstrated the presence of \( \beta_1, \beta_2, \) and \( \alpha_1, \alpha_2 \) integrins in myocytes as well as non-myocytes and \( \alpha_1 \) only in non-myocytes in our cultures. Inclusion of antibodies directed against \( \beta_1, \beta_2, \) or \( \alpha_1, \alpha_2, \) or cadherin, was effective in blocking the BNP promoter response to mechanical strain. These same antibodies (anti-\( \beta_1, \beta_2, \) and -\( \alpha_1, \alpha_2 \)) had a similar inhibitory effect on strain-stimulated ERK, p38 MAPK, and, to a lesser extent, JNK activities in these cells. Cotransfection with chimeric integrin receptors capable of acting as dominant-negative inhibitors of integrin function demonstrated suppression of strain-dependent BNP promoter activity when vectors encoding \( \beta_1, \alpha_5, \) but not \( \alpha_1, \alpha_5, \) or a carboxyl-terminal deletion mutant of \( \beta_2 (\beta_2B), \) were employed. These studies underscore the importance of cell-matrix interactions in controlling cardiac gene expression and suggest a potentially important role for these interactions in signaling responses to mechanical stimuli within the myocardium.

Brain natriuretic peptide (BNP) is a vasoactive hormone that, despite its name, is produced primarily in the heart. Like atrial natriuretic peptide (ANP), it possesses potent natriuretic, diuretic, and vasorelaxant activities, properties that position it physiologically as a potential antagonist of vasoactive systems (e.g. the renin-angiotensin and sympathetic nervous systems) associated with intravascular volume expansion and increased blood pressure (1).

Like ANP, BNP is expressed in both atrial and ventricular myocardia, although differential expression is not so marked as that for ANP (the atrial/ventricular ratio for BNP expression is ~3:1, whereas that for ANP is in the range of 40:1) (2). As with ANP, ventricular expression of BNP is activated in pathophysiological states associated with hemodynamic overload in vivo (3, 4) and following exposure to hypertrophy-promoting maneuvers in vitro (5–10). In the latter group are a number of biochemical (e.g. \( \alpha \)-adrenergic agonists, endothelin, angiotensin II, and cardiotrophin-1) as well as physical (e.g. mechanical strain and hypoxia) stimuli that trigger increases in cell size and protein synthesis, reactivate a program of fetal gene expression, and reorganize sarcomeric structure of myocytes in culture. These phenotypic changes closely resemble those associated with myocyte hypertrophy in vivo (11).

A number of recent studies have documented the importance of the extracellular matrix in establishing cellular responses, both qualitatively and quantitatively, to a variety of biochemical and physical stimuli (12, 13). In the case of mechanical strain, it has been hypothesized that key matrix-integrin attachments may participate in the signal transduction process linking the strain signal to changes in gene expression, cytoskeletal reorganization, and DNA synthesis (14).

We have recently shown that application of cyclical, passive mechanical strain (i.e. stretch) to cultured neonatal rat ventricular myocytes in vitro results in stimulation of immunoreactive BNP secretion, increased steady-state levels of the BNP gene transcript, and activation of a transfected human BNP (hBNP) gene promoter (8). In the present study, we show that both basal and strain-activated BNP promoter activities are dependent upon specific contacts that the ventricular myocyte makes with proteins in the extracellular matrix, implying a potential signaling function for these contacts in the subsequent activation of gene expression.

EXPERIMENTAL PROCEDURES

Materials—Fibronectin was purchased from Sigma. Peptides GRGDS and GRGES (amino acids are designated by conventional single-letter nomenclature) were purchased from Life Technologies, Inc. FLEX plates were from Flexcell International Corp. (McKeesport, PA). Polyclonal antibodies directed against ERK, JNK, and p38 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Protein G-Sepharose was purchased from Amersham Pharmacia Biotech. [\( \gamma^{32} \text{P} \)]ATP was purchased from NEN Life Science Products. Bovine myelin basic protein was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Glutathione S-transferase-c-Jun was prepared as described (15). Purified hamster anti-rat/mouse \( \alpha_1, \alpha_2, \alpha_5, \) and \( \beta_1 \) monoclonal antibodies; purified mouse anti-\( \beta_1, \beta_2, \) monoclonal antibody; FITC-labeled anti-hamster IgG; and FITC-conjugated hamster anti-rat \( \beta_1 \) monoclonal antibody were obtained from Pharmingen (San Diego, CA).
CA). Anti-pan cadherin monoclonal antibody was purchased from Sigma. Anti-α, β1, β3, and phycocerythrin-labeled anti-mouse IgG were provided by Dean Sheppard (University of California, San Francisco). Other reagents were obtained though standard commercial suppliers.

Plasmid Constructions—The construction of −1595 hBNP-PLUC has been described previously (16). Cytomegalovirus-driven chimeric β3, β2, βB, β3, and α1 integrin expression vectors were provided by Susan E. LaFlamme (Albany Medical College, Albany, NY) (17, 18). These vectors link the DNA sequence encoding the intracellular domains of the individual integrins to the extracellular and transmembrane domains of the human interleukin-2 receptor (gp55 subunit). Plasmid containing the Rous sarcoma virus-β-galactosidase reporter was provided by Wen-Feng Wang (University of California, San Francisco).

Cell Culture and Application of Mechanical Strain—Ventricular myocytes were prepared from 1–2-day-old neonatal rat hearts by alternate cycles of 0.05% tryptic digestion and mechanical disruption as described previously (19). Cells (1 × 10⁶) were cultured on collagen-coated FLEX plates in Dulbecco’s modified Eagle’s/H-21 medium containing 10% bovine calf serum (Hyclone Laboratories, Logan, UT), 2 mM glutamine, 10 units/ml penicillin, and 100 mg/ml streptomycin. A glass cloning cylinder (1-cm diameter) was placed in the middle of each well to preclude cell attachment, thereby placing the majority of adherent cells on the outer 75% of the culture surface where distension is maximal. Flexcell Strain apparatus was applied to apply strain to our cultured myocytes. This is a commercially marketed device that applies vacuum (at variable levels) to the undersurface of a collagen-coated silicone elastomer disc positioned in the bottom of the well culture dish, which, in turn, is secured in an airtight manifold. Computer-regulated application of negative pressure to the underside of the culture dish results in downward displacement of the disc and stretch of adherent cells cultured on its upper surface (21–24). The medium was changed 24 h prior to initiation of the experiment. Cells were subjected to cyclical strain (60 cycles/min) on the Flexcell Strain apparatus at a level of distension sufficient to promote ~20% increment in surface area at the point of maximal distension on the culture surface (20). Preliminary experiments indicated that this level of distension provided near-maximal induction of BNP gene promoter activity (data not shown).

Transfection and Luciferase and β-Galactosidase Assays—Freshly prepared ventricular myocytes were transiently transfected with the indicated reporters and expression vectors by electroporation (Gene Pulser, Bio-Rad) at 280 mV and 250 microfarads. DNA content in individual cultures was normalized with pUC18. After transfection, cells were plated and cultured as described above. Cells were harvested and prepared 48 h after transfection for luciferase activity assay. Protein concentration of each cell extract was measured using Coomassie blue dye. Cytomegalovirus-driven chimeric β1 integrins were not demonstrated in the myocyte cultures. Fibronec-tin, one of the major matrix proteins in the myocardium (25, 26), has been shown to exert profound effects on gene expression in other systems (27, 28). With this in mind, we explored the effects of exogenous fibronec-tin on the activity of the BNP gene promoter in our myocyte cultures. When culture surfaces were precoated with increasing concentrations of fibronec-tin, a dose-dependent increase in basal hBNP luciferase activity and amplification of the strain-dependent response (Fig. 1A) were observed, implying that interaction of the myocyte with this matrix component is an important determinant of the magnitude of the response to mechanical strain.

Disruption of cell-matrix interactions in the myocyte cultures negatively impacted the magnitude of the BNP promoter response to strain. Addition of a soluble peptide containing the matrix protein sequence RGD (i.e. GRGDSP) to the myocyte cultures, a maneuver that is known to interfere with selected matrix-integrin interactions, effected a dose-dependent reduction in strain-stimulated BNP gene promoter activity (Fig. 1B). When the same experiment was carried out with the functionally inert RGE peptide (i.e. GRGESP), no reduction in promoter activity was observed. Similar inhibition was observed when soluble fibronec-tin was included in the culture medium (Fig. 1C). As with the RGD peptide, a dose-dependent reduction in BNP promoter activity was seen, presumably reflecting impaired generation of matrix-integrin contacts in the cultures. Collectively, these findings imply that intact matrix-integrin attachments are required for strain-dependent induction of BNP gene transcription.

Next, we examined the nature of the integrins expressed in our cultured cells using antibody-driven fluorescence-activated cell sorting analysis. As shown in Fig. 2, we detected the presence of β1, β3, and α3β1 integrins in our primary cultures of ventricular myocytes. Since these cultures are routinely contaminated to some degree with non-myocytes (primarily fibroblasts), we examined the integrin profile in myocytes cultured in the presence of bromodeoxyuridine. In addition, we examined the profile in non-myocytes (predominantly fibroblasts), we reexamined the integrin profile in myocyte cultures revealed differential effects on hBNP promoter activity. As shown in Fig. 3, antibodies against β1, β3, and α3 integrins; α1 integrins were not demonstrated in the myocyte cultures.

Addition of these same antibodies to viable transfected myocyte cultures revealed differential effects on hBNP promoter activity. As shown in Fig. 3, antibodies against β1, β3, and α3 integrins clearly inhibited the promoter response to mechanical strain.
Antibody against the α5 integrin effected a modest reduction in the response to strain, whereas antibody against α1, α2, or cadherin was without effect. These results were supported by additional studies employing transient overexpression of chimeric integrin molecules that link the extracellular and transmembrane domains of interleukin-2 to the intracellular domains of different integrin monomeric proteins. The latter, in the absence of a bona fide extracellular domain, do not respond to stimuli requiring engagement of the extracellular matrix, but continue to associate with and compete for intracellular proteins responsible for signaling integrin-dependent activity. In effect, when the chi-

Antibody against the α5 integrin effected a modest reduction in the response to strain, whereas antibody against α1, α2, or cadherin was without effect.

These results were supported by additional studies employing transient overexpression of chimeric integrin molecules that link the extracellular and transmembrane domains of interleukin-2 to the intracellular domains of different integrin monomeric proteins. The latter, in the absence of a bona fide extracellular domain, do not respond to stimuli requiring engagement of the extracellular matrix, but continue to associate with and compete for intracellular proteins responsible for signaling integrin-dependent activity. In effect, when the chi-
In the cardiovascular system, the extracellular matrix is known to play an important role in modulating cellular function (12, 32, 33). In addition, because of the dynamic (i.e. pulsatile) environment in which these cells reside, considerable interest has focused on the possibility that formation/disruption of matrix attachments play a major role in morphogenesis. It is also important in differentiated cells where physiological function is dependent upon specific cell-matrix attachments (e.g. leukocyte adhesion to blood vessel walls or osteoclast attachment to remodeling bone surfaces).

The major findings of this study include demonstration that 1) matrix proteins, including fibronectin, play an important role in supporting both basal and strain-induced BNP gene transcription in ventricular myocytes; 2) selected integrins present on the surface of cardiac myocytes participate in the activation of a number of signaling pathways that have been linked to the strain response as well as myocyte hypertrophy; and 3) interference with the function of these integrins leads to suppression of strain-dependent BNP gene transcription.

A number of integrins have been identified previously on cardiac cells or in myocardial tissue: \( \beta_1 \) (33, 34), \( \beta_2 \) (35), \( \alpha_3 \) (34), \( \alpha_5 \) (34), and \( \alpha_5 \beta_1 \) (36) integrins have been shown to be present on cardiac myocytes, whereas \( \alpha_5 \) (37), \( \alpha_6 \) (38), and \( \alpha_6 \beta_5 \) (37) have been identified in rat or human fibroblasts cultured from either neonatal or adult hearts. It is noteworthy that \( \alpha_1 \),
A

\begin{figure}[h]
\centering
\includegraphics[width=\linewidth]{figure5a.png}
\caption{Effect of anti-integrin antibodies on strain-induced ERK, JNK, and p38 activities in neonatal rat ventricular myocytes. After 48 h of culture, cells were treated with 1 \(\mu\)g of antibody directed against \(\beta_1\) or \(\beta_3\) integrin, a 1:10 dilution of antiserum directed against \(\alpha_5\beta_1\) integrin, or 1 \(\mu\)g of antibody against cadherin for at least 1 h and then subjected to strain for 15 min. Cells were collected and assayed for ERK (A), JNK (B), or p38 (C) activity as described under "Experimental Procedures." Pooled data are presented as means ± S.D. from three separate experiments. * , \(p < 0.01\) versus strain control.}
\end{figure}

\section*{Strain-activated BNP Promoter}

The presence of \(\beta_1\), \(\beta_3\), and \(\alpha_5\beta_3\) integrins in both myocytes and non-myocytes in our cultures raises the obvious possibility that the antisera or dominant-negative mutants might be target Integrins present on the non-myocytes, secondarily suppressing a paracrine activator of BNP gene expression in the myocytes. Such paracrine activators exist (39, 40); however, they account, at best, for ~50% of the hBNP promoter response to mechanical strain. The fact that the inhibition with anti-integrin antisera exceeded 50% implies that a significant portion of the integrin-dependent activity operates directly at the level of the cardiac myocyte. Furthermore, we have employed the same anti-integrin antibodies in cultures depleted of non-myocytes through treatment with bromodeoxyuridine, and we continue to see near-total inhibition of the response to strain (data not shown), again supporting an effect directly at the level of the cardiac myocyte.

It appears that activation of the hBNP gene promoter in our cultures is heavily dependent upon interaction of cellular integrins with fibronectin or fibronectin-like proteins in the extracellular matrix. Both the RGD peptide, which harbors a soluble integrin-binding site, and soluble fibronectin itself inhibited the response to strain, presumably by competing for matrix attachments. Morphologically, the cells appeared normal in each case and continued to adhere to the culture surface, indicating that attachment to the surface alone does not confer sensitivity to strain and implying that formation and/or disruption of selected matrix attachments may trigger specific signals that lead to changes in cellular phenotype. Although fibronectin clearly appears to be important, other matrix proteins (e.g., vitronectin and collagen) also utilize the RGD recognition sequence for integrin association. In addition, cardiac myocytes under hypertrophy-promoting conditions have been shown to produce fibronectin, vitronectin, and collagens I and III (26, 41); and rat cardiac fibroblasts have been shown to increase synthesis of fibronectin and collagen III in response to biaxial stretch (42). Thus, one or more of these matrix proteins may establish the key integrin attachments that are required to signal the response to strain.

Ingber (43) has suggested that generation of tension in the matrix-integrin-cytoskeletal network activates the signaling cascade(s) that lead to changes in cell shape, mitogenesis, and gene expression. The fact that functional neutralization of several different integrins (through exogenous antibodies or transfected dominant-negative mutants) effected a substantial reduction of the hBNP transcriptional response to strain supports the notion that tension within the matrix-integrin-cytoskeletal assembly per se drives the response, presumably through integrin-dependent signal transduction pathways. The data would suggest that there is a quantitative threshold of integral cellular tension that must be maintained to support the transcriptional response. If this threshold is not maintained, the relevant signaling mechanisms fire imperfectly or

\(\alpha_5\) and \(\beta_1\) integrins are up-regulated after induction of cardiac hypertrophy in the adult rat (34). \(\beta_3\) integrin has been linked to assembly of a Src signaling complex (along with focal adhesion kinase) during ventricular hypertrophy \textit{in vivo} (35). \(\beta_3\) has also been implicated as playing an important role in cardiac development (32).

We have identified \(\beta_1\), \(\beta_3\), and \(\alpha_5\beta_3\) on both myocytes and fibroblasts in our cultures. Of note, unlike MacKenna et al. (38), we did not identify \(\alpha_5\) in our fibroblast cultures. This could reflect differences in the integrin expression profile in the two experimental systems or, alternatively, limited efficacy of our anti-\(\alpha_5\) antibody to interact with the surface integrin. Of note, high concentrations of our anti-\(\alpha_5\) antibody appeared to effect a modest reduction in strain-dependent BNP promoter activity, implying that at least small amounts of this integrin are present in our cultures.

The presence of \(\beta_1\), \(\beta_3\), and \(\alpha_5\beta_3\) integrins on both myocytes and non-myocytes in our cultures raises the obvious possibility that the antisera or dominant-negative mutants might be targeting integrins present on the non-myocytes, secondarily suppressing a paracrine activator of BNP gene expression in the myocytes. Such paracrine activators exist (39, 40); however, they account, at best, for ~50% of the hBNP promoter response to mechanical strain. The fact that the inhibition with anti-integrin antisera exceeded 50% implies that a significant portion of the integrin-dependent activity operates directly at the level of the cardiac myocyte. Furthermore, we have employed the same anti-integrin antibodies in cultures depleted of non-myocytes through treatment with bromodeoxyuridine, and we continue to see near-total inhibition of the response to strain (data not shown), again supporting an effect directly at the level of the cardiac myocyte.

It appears that activation of the hBNP gene promoter in our cultures is heavily dependent upon interaction of cellular integrins with fibronectin or fibronectin-like proteins in the extracellular matrix. Both the RGD peptide, which harbors a soluble integrin-binding site, and soluble fibronectin itself inhibited the response to strain, presumably by competing for matrix attachments. Morphologically, the cells appeared normal in each case and continued to adhere to the culture surface, indicating that attachment to the surface alone does not confer sensitivity to strain and implying that formation and/or disruption of selected matrix attachments may trigger specific signals that lead to changes in cellular phenotype. Although fibronectin clearly appears to be important, other matrix proteins (e.g., vitronectin and collagen) also utilize the RGD recognition sequence for integrin association. In addition, cardiac myocytes under hypertrophy-promoting conditions have been shown to produce fibronectin, vitronectin, and collagens I and III (26, 41); and rat cardiac fibroblasts have been shown to increase synthesis of fibronectin and collagen III in response to biaxial stretch (42). Thus, one or more of these matrix proteins may establish the key integrin attachments that are required to signal the response to strain.

Ingber (43) has suggested that generation of tension in the matrix-integrin-cytoskeletal network activates the signaling cascade(s) that lead to changes in cell shape, mitogenesis, and gene expression. The fact that functional neutralization of several different integrins (through exogenous antibodies or transfected dominant-negative mutants) effected a substantial reduction of the hBNP transcriptional response to strain supports the notion that tension within the matrix-integrin-cytoskeletal assembly per se drives the response, presumably through integrin-dependent signal transduction pathways. The data would suggest that there is a quantitative threshold of integral cellular tension that must be maintained to support the transcriptional response. If this threshold is not maintained, the relevant signaling mechanisms fire imperfectly or
not at all, thereby abolishing the response. Numerous signal transduction pathways have been shown to activate in response to integrin ligation, including Ras (44), non-receptor tyrosine kinases (e.g. Src and Fyn) (45, 46, 53), focal adhesion kinase (45, 47, 53), ERK (46, 48–50), JNK (47, 50), and p38 MAPK (51). Importantly, we have show previously that ERK, JNK, and p38 MAPK are each activated following application of strain to our myocyte cultures (8, 29), and each of them appears to play an important role in mediating the transient signaling response to strain. Clerk et al. (30) have noted similar activation of these three pathways following α-adrenergic induction of neonatal rat cardiac myocytes, providing support for a shared role in controlling downstream transcriptional activity. This suggests that there may be a functional interaction among these pathways that requires that all be operative to achieve an optimal response. It is noteworthy that MacKenna et al. (38) found that stretch activated ERK and JNK, but not p38 MAPK, in adult rat cardiac fibroblasts. In this case, the ERK response was mediated by RGD-directed, integrin-dependent activity, whereas the JNK response was not. The latter finding stands in contrast to our findings, implying important differences in the myocyte versus non-myocyte integrin-dependent signal transduction pathways. It is clear, however, that strain-dependent induction of JNK was only partially inhibited by integrin neutralization. Li et al. (52) reported that although shear stress induction of ERK activity in bovine aortic endothelial cells was completely blocked by inhibition of the integrin-associated focal adhesion kinase, the induction of JNK was only partially blocked. This may suggest that activation of ERK and possibly p38 involves an integrin/ focal adhesion kinase-signaled event, whereas JNK is activated by this as well as a second focal adhesion kinase-independent pathway.

In summary, we have shown that strain-dependent increments in hBNP gene promoter activity are critically dependent on specific matrix-integrin attachments established at the cell periphery. It is conceivable that perturbation of these attachments may serve as the primary stimulus that the cell senses in response to cellular deformation. Processing of this signal through the integrins, cytoskeletal proteins, or the various proteins associated with these structures (43) presumably triggers the multiple cellular events that are evoked in response to strain. Careful elucidation of the individual molecular participants in this process may provide us with a better understanding of the cellular and molecular events linking hemodynamic overload to cardiac hypertrophy.

Acknowledgments—We are grateful to Karl Nakamura and Fred Roediger for assistance with cell preparation and to Drs. E. Wilson, D. Sheppard, S. LaFlamme, and W. Feng for helpful discussions and reagents used in this study.

REFERENCES

1. Kamabayshi, Y., Nakao, K., Kimura, H., Kawabata, T., Nakamura, M., Inouye, K., Yoshida, N., and Imura, H. (1999) Biochem. Biophys. Res. Commun. 263, 599–605
2. Gerbes, A. L., Dagnino, L., Nguyen, T., and Nemer, M. (1994) J. Clin. Endocrino. Metab. 78, 1397–1311
3. Kohno, M., Horio, T., Yoshiyama, M., and Takeda, T. (1992) Hypertension 19, 206–211
4. Hasegawa, K., Fujiiwara, H., Doymaz, M., Miyamoto, M., Fujiiwara, T., Suga, S., Mukoyama, M., Nakao, K., Imura, H., and Hayaizama, S. (1993) Circulation 88, 372–380
5. Nakagawa, O., Ogawa, Y., Itoh, M., Suga, S., Komatsuzaki, Y., Kishimoto, I., Nishino, K., Yoshimasa, T., and Nakao, K. (1995) J. Clin. Invest. 96, 1280–1287
6. Harada, M., Itoh, H., Nakagawa, O., Ogawa, Y., Miyamoto, Y., Kuwahara, K., Ogawa, E., Igi, T., Yashimoto, J., Masuda, I., Yoshimasa, T., Tanaka, I., Saito, Y., and Nakao, K. (1997) Circulation 96, 7375–7379
7. Hanford, D. S., Theurer, D. J., Murray S. F., and Glembocksi, C. C. (1994) J. Biol. Chem. 269, 26227–26233