RhoA/ROCK signaling is critical to FAK activation by cyclic stretch in cardiac myocytes

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Torsoni, Adriana S., Talita M. Marin, Licio A. Velloso, and Kleber G. Franchini. RhoA/ROCK signaling is critical to FAK activation by cyclic stretch in cardiac myocytes. Am J Physiol Heart Circ Physiol 289: H1488–H1496, 2005. First published May 27, 2005; doi:10.1152/ajpheart.00692.2004.—Focal adhesion kinase (FAK), a tyrosine kinase linked to integrin signaling (15, 24, 42), has been shown to be rapidly activated by mechanical stimuli in cardiac myocytes (2, 5, 9, 12, 13, 23, 34, 39). Several lines of evidence support a role for FAK in the regulation of early gene transcription in response to hypertrophic agonists and mechanical stress (10, 22, 28, 38, 39), indicating that this kinase may coordinate the convergence of multiple signaling pathways involved in the hypertrophic growth of cardiac myocytes. However, the molecular mechanism responsible for FAK activation by mechanical stress in cardiac myocytes remains elusive. We recently showed (12, 39) that FAK activation by mechanical stress is accompanied by its aggregation at myofilaments, Z disks, and costameres, implying that this kinase might be directly activated by mechanical stress in cardiac myocytes. On the other hand, FAK activation in neonatal rat ventricular myocytes (NRVMs) by agonists such as endothelin has been demonstrated (16) to be dependent on activation of the RhoA/Rho-associated coiled-coil-containing protein kinase (ROCK) signaling pathway, which drives the assembly and rearrangement of actin filaments. The demonstration that cytochalasin D, a potent inhibitor of actin polymerization, markedly attenuated endothelin-induced FAK phosphorylation (16) revealed the importance of the assembly of actin filaments in FAK activation by this agonist. Similarly, FAK activation by mechanical stress has been suggested to depend on a cooperative interaction with actin filament (13, 39, 40), raising the possibility that FAK activation by mechanical stimuli may also be dependent on RhoA/ROCK signaling. Further support for this idea is provided by data (20, 40) indicating that RhoA and ROCK, similar to FAK, are rapidly but transiently activated by mechanical stress in cardiac myocytes. However, the role of RhoA/ROCK in activation of FAK by mechanical stress in cardiac myocytes has not been established.

Thus, to gain insight into the activation of FAK by mechanical stress in cardiac myocytes, we tested the hypothesis that the RhoA/ROCK signaling pathway plays a role in stretch-induced FAK activation in NRVMs. By preventing RhoA/ROCK signaling through pharmacological inhibition with C3 exoenzyme or transfection with the RhoA antisense oligonucleotide or the ROCK-specific inhibitor Y-27632, we showed that RhoA/ROCK signaling is critical to stretch-induced FAK activation as well as to regulation of stretch-induced expression of β-myosin heavy chain (β-MHC) in cardiac myocytes.

INCREASED BIOMECHANICAL STRESS can drive changes in cardiac myocytes that are implicated in myocardial hypertrophy and failure (7, 19). Numerous signal transduction pathways have been shown to be activated in cardiac myocytes subjected to mechanical stimuli (35). Signals originating from multiple pathways converge intracellularly, leading to altered gene expression and protein synthesis, which result in the hypertrophic growth of cardiac myocytes. However, the mechanism by which mechanical forces are sensed and converted to biochemical signals remains largely unknown. Recent developments in this field indicate that the integrity of structures such as the Z disk, costamere, and intercalated disk is critically important to the ability of cardiac cells to appropriately respond to mechanical stress (4, 11, 21, 32, 37, 41). It has been hypothesized that such structures participate in monitoring of mechanical force and in communication of strain to signaling molecules in cardiac myocytes (12, 29, 37).

Focal adhesion kinase (FAK), a tyrosine kinase linked to integrin signaling (15, 24, 42), has been shown to be rapidly activated by mechanical stimuli in cardiac myocytes (2, 5, 9, 12, 13, 23, 34, 39). Several lines of evidence support a role for FAK in the regulation of early gene transcription in response to hypertrophic agonists and mechanical stress (10, 22, 28, 38, 39), indicating that this kinase may coordinate the convergence of multiple signaling pathways involved in the hypertrophic growth of cardiac myocytes. However, the molecular mechanism responsible for FAK activation by mechanical stress in cardiac myocytes remains elusive. We recently showed (12, 39) that FAK activation by mechanical stress is accompanied by its aggregation at myofilaments, Z disks, and costameres, implying that this kinase might be directly activated by mechanical stress in cardiac myocytes. On the other hand, FAK activation in neonatal rat ventricular myocytes (NRVMs) by agonists such as endothelin has been demonstrated (16) to be dependent on activation of the RhoA/Rho-associated coiled-coil-containing protein kinase (ROCK) signaling pathway, which drives the assembly and rearrangement of actin filaments. The demonstration that cytochalasin D, a potent inhibitor of actin polymerization, markedly attenuated endothelin-induced FAK phosphorylation (16) revealed the importance of the assembly of actin filaments in FAK activation by this agonist. Similarly, FAK activation by mechanical stress has been suggested to depend on a cooperative interaction with actin filament (13, 39, 40), raising the possibility that FAK activation by mechanical stimuli may also be dependent on RhoA/ROCK signaling. Further support for this idea is provided by data (20, 40) indicating that RhoA and ROCK, similar to FAK, are rapidly but transiently activated by mechanical stress in cardiac myocytes. However, the role of RhoA/ROCK in activation of FAK by mechanical stress in cardiac myocytes has not been established.

Thus, to gain insight into the activation of FAK by mechanical stress in cardiac myocytes, we tested the hypothesis that the RhoA/ROCK signaling pathway plays a role in stretch-induced FAK activation in NRVMs. By preventing RhoA/ROCK signaling through pharmacological inhibition with C3 exoenzyme or transfection with the RhoA antisense oligonucleotide or the ROCK-specific inhibitor Y-27632, we showed that RhoA/ROCK signaling is critical to stretch-induced FAK activation as well as to regulation of stretch-induced expression of β-myosin heavy chain (β-MHC) in cardiac myocytes.
MATERIALS AND METHODS

Reagents. Bioflex (Flex I) collagen culture plates were obtained from Flexcell International (catalog no. 35-P-1001C, type I); protein A conjugated with 125I from Du Pont-New England Nuclear; Dulbecco’s modified Eagle’s medium (DMEM), horse serum, and fetal bovine serum from GIBCO BRL; pancreatin and angiotensin II (ANG II) from Sigma; collagenase type II from Worthington; rabbit polyclonal antibodies to FAK and ERK1/2 and mouse monoclonal RhoA antibody from Santa Cruz Biotechnology; rabbit polyclonal antibodies to FAK phosphorylated at Tyr397 and ERK1/2 phosphorylated at Thr202/Tyr204 from Biosource International; and cytochalasin D, Clostridium botulinum C3 exoenzyme, Y-27632 (an ROCK inhibitor), and DuP-753 from Calbiochem. Oligonucleotides were synthesized by Life Technologies.

NRVM culture. Primary cultures of NRVMs from 1- to 2-day-old Wistar rats were prepared as previously reported (39). Briefly, the myocytes were purified on a discontinuous Percoll gradient, suspended in plating medium containing 10% horse serum, 5% fetal bovine serum, and 0.5% penicillin-streptomycin, and plated in type I collagen Bioflex plates coated with gelatin at 5 x 10^4 cells/well. After 24 h, the medium was replaced with serum-free DMEM and incubated for 24 – 48 h under 95% air-5% CO2 before use. NRVMs cultured in Bioflex plates were stretched in a Flexcell FX-3000 strain unit to 115% of resting length at a frequency of 1 Hz (0.5 s of stretch and 0.5 s of relaxation) for variable periods, depending on the protocol. Control nonstretched NRVMs were also cultured in Bioflex plates and incubated in serum-free medium. At the conclusion of the experimental protocols, cells were scraped from membranes and lysed for immunoblotting for analysis of β-MHC expression or fixed for confocal immunofluorescence analysis.

Immunoblotting. NRVMs were lysed in assay lysis buffer containing freshly added protease and phosphatase inhibitors (1% Triton, 10 mM sodium pyrophosphate, 100 mM NaF, 10 μg/ml aprotinin, 1 mM PMSF, and 0.25 mM Na3VO4). The samples were centrifuged for 20 min at 11,000 g, and the soluble fraction was resuspended in 50 μl of Laemmli loading buffer (2% SDS, 20% glycerol, 0.04 mg/ml bromophenol blue, 0.12 M Tris-HCl, pH 6.8, and 0.28 M β-mercaptoethanol) before separation on 8% SDS-polyacrylamide gels. Proteins were transferred from the gels to a nitrocellulose membrane. Membranes were blocked for 2 h at room temperature with 5% skim milk-TBST buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.05% Tween 20). Membranes were exposed to primary antibodies overnight at 4°C, washed in TBST, and exposed to 125I-labeled protein A.

Subcellular fractionation. Subcellular fractionation was carried out as previously described (12) with modifications for use with NRVMs. Briefly, NRVMs were collected in solubilization buffer (0.32 M saccharose, 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1 mM DTT, 50 mM NaH2PO4, 1 mM Na3VO4, 2 mM PMSF, and 0.1 mg/ml aprotinin) and homogenized by 10 passages of the cell suspension through a 26.5-gauge needle. The extracts were centrifuged at 1,000 g for 10 min at 4°C to obtain the precipitates containing the nuclear fraction. The supernatant, which was enriched with membrane, myofibrils, and soluble proteins, was saved. Proteins of nuclear and supernatant fractions were quantified by the Bradford method.

Fig. 1. Cyclic stretch-induced focal adhesion kinase (FAK) and ERK1/2 phosphorylation. Neonatal rat ventricular myocytes (NRVMs) were stretched to 115% for 10, 30, 60, and 120 min at 1 Hz. A: Western blots of whole cell extracts probed with antibodies that recognize RhoA, FAK phosphorylated at Tyr397 (pFAK), unphosphorylated FAK, ERK1/2 phosphorylated at Thr202/Tyr204 (pERK1/2), and unphosphorylated ERK1/2. B: densitometric analysis of 4 Western blot experiments performed with antibodies against pFAK and pERK1/2 normalized by total FAK and ERK1/2, respectively. C: representative immunoblot (IB) and results from scanning densitometry showing average (4 experiments) percent changes in amount of pERK1/2 in ANG II-treated cells compared with control (c). D: representative blots probed with pERK1/2 antibody and average results from densitometric analysis of NRVMs treated with ANG II (2.7 μM/l) + DuP-753 (DuP, 10 μM/l) and stretched to 115% for 30 min. *P < 0.05 compared with nonstretched cells.
Equal amounts of total protein were denatured after addition of Laemmli buffer and boiling at 100°C for 5 min and subjected to SDS-PAGE.

**Oligodeoxynucleotides.** FAK antisense oligodeoxynucleotide (ODN) was a 16-mer (5'-GATAAGCAGCTGCCAT-3') directed against initiation of the translation site of the rat FAK mRNA sequence. The FAK sense sequence (5'-CGCTAACCAGTGTA-3') was used as control. RhoA antisense ODN was a 16-mer (5'-TCCTGATGGCAGCCAT-3') directed against initiation of the translation site of the rat RhoA mRNA sequence. The RhoA sense sequence (5'-GTTAATCTTGACAGTA-3') was used as control. All bases were phosphorothioate protected and obtained from Life Technologies. The sequences were confirmed in the GenBank database for uniqueness. Transfections of NRVMs with ODNs were performed as previously described (26). Cells were serum starved for 6 h and transfected with 1 μmol/l antisense or sense ODN and 12 μg of Lipofectin in serum-starved DMEM without antibiotics (1 ml final volume) for 6 h. NRVMs were washed with DMEM and maintained in DMEM containing 10% serum for 18 h before use in experiments.

**RT-PCR analysis.** NRVMs were homogenized in TRIzol reagent, and total RNA was isolated by precipitation with isopropyl as previously described (26). A 5-μg aliquot of total RNA was used for cDNA synthesis with the Superscript preamplification system (Life Technologies) according to the manufacturer’s instructions. cDNA was amplified by PCR using Taq DNA polymerase with oligonucleotides derived from the β-MHC gene (5'-CCAACACTCACCTGTCAAGTGC-3' and 5'-TGCAGGCTCCAGGGTCG-3') or β-actin gene (5'-TTCTACAATGAGCTGCTG-3' and 5'-GCTTCTCCCTTAATGTCACGA-3'). Oligonucleotides were synthesized by Life Technologies. The amplification conditions consisted of 25 cycles as follows: denaturing at 94°C for 2 min, annealing at 45°C (β-actin) and 54°C (β-MHC) for 1 min, and extension at 72°C for 2 min. PCR products were size fractionated with agarose gel electrophoresis. After the gels were stained with ethidium bromide, the DNA bands were visualized with a UV transilluminator.

**Immunohistochemistry and laser confocal analysis.** NRVMs were fixed with 4% paraformaldehyde and 4% sucrose in 0.1 mol/l phosphate-saline buffer, pH 7.4, for 15 min at room temperature. The slides were preincubated in blocking buffer (3% nonfat dry milk in 0.1 mol/l PBS) containing 0.6% Triton X-100 for 45 min at room temperature and incubated overnight with the primary monoclonal RhoA antibody and then with rabbit anti-mouse biotin-conjugated secondary antibody and streptavidin-Cy2 (1:500 in PBS) and rhodamine-conjugated phalloidin at 4°C. Positive immunoreactivity was visualized by laser confocal scanning (Zeiss LM510). In the absence of primary antibodies, application of secondary antibodies (negative controls) failed to produce any significant staining.

**Statistical analysis.** Data are presented as percent changes compared with controls. Densitometric readings were subjected to statistical analysis. Differences between the mean values of the densitometric readings were tested by ANOVA and Bonferroni’s multiple-range test. P < 0.05 indicated statistical significance.

**RESULTS**

Cyclic stretch induces FAK and ERK1/2 activation in NRVMs. Cardiac myocytes were extracted from 1- to 2-day-old neonatal rats and cultured on silicone plates for 72 h before they were subjected to cyclic stretch (to 115% at 1 Hz) for 10–120 min. Cyclic stretch of NRVMs induced rapid (10 min) and sustained (up to 120 min) FAK and ERK1/2 activation (Fig. 1A), as indicated by increases in the amount of FAK and
ERK1/2 detected by specific antibodies against FAK phosphorylated at Tyr397 and ERK1/2 phosphorylated at Thr202/Tyr204. The amount of FAK, ERK1/2, and RhoA did not change in stretched compared with nonstretched NRVMs. The percent changes in phosphorylation of FAK at Tyr397 and ERK1/2 at Thr202/Tyr204 normalized by the amount of FAK and ERK1/2, respectively, are summarized in Fig. 1B.

ANG II may act as an autocrine/paracrine mediator of stretch-induced cardiomyocyte hypertrophy (1, 31). FAK and RhoA have been shown to be activated in response to G protein-coupled receptor agonists, including ANG II (10, 16, 33). We recently showed that FAK phosphorylation induced by cyclic stretch still activates FAK in NRVMs treated with the AT1 receptor antagonist DuP-753, indicating that stretch and ANG II activate FAK by distinct mechanisms (39). We examined whether ANG II mediates the activation of ERK1/2 phosphorylation at Thr202/Tyr204 induced by cyclic stretch in NRVMs. ANG II induced concentration-dependent increases of ERK1/2 phosphorylation in NRVMs (Fig. 1C). This effect was completely inhibited by addition of the AT1 receptor-
specific antagonist DuP-753 (10 μmol/l, 1 h) to the culture medium, indicating that ANG II-induced ERK1/2 phosphorylation occurred via AT1 receptor-dependent signaling (Fig. 1D). Cyclic stretch still increased ERK1/2 phosphorylation at Thr202/Tyr204 in DuP-753-treated NRVMs, although it was markedly attenuated compared with the response in untreated cells (Fig. 1D).

**FAK and RhoA are associated in nonstretched NRVMs.** FAK phosphorylation by 30 min of cyclic stretch is accompanied by its aggregation at myofilaments of NRVMs (39). We examined RhoA localization and distribution in nonstretched and stretched NRVMs. A representative example of NRVMs double stained with RhoA antibody and rhodamine-conjugated phalloidin is shown in Fig. 2, A–C. Anti-RhoA staining shows a sarcomeric striated pattern alternated with actin labeling, suggesting its localization in the A bands or the nearby structures. In stretched cells, anti-RhoA staining still showed a sarcomeric striated pattern alternated with actin labeling, but a distinct staining of nuclear or perinuclear areas was also observed. To further examine the subcellular distribution of RhoA in NRVMs, we performed immunoblotting analysis of subcellular fractions. RhoA could not be detected in the nuclear fraction of nonstretched or stretched cells (Fig. 3A), indicating that the anti-RhoA staining in the nuclear area of stretched NRVMs is probably due to a perinuclear location.

The association of RhoA and FAK was indicated by the demonstration that, in extracts of nonstretched NRVMs, FAK is coimmunoprecipitated with RhoA. Cyclic stretch lasting for 120 min was accompanied by a reduction in the association of FAK with RhoA (Fig. 3B). Comparisons with the amount of RhoA precipitated with RhoA antibody allow us to estimate that ~60% of RhoA is associated with FAK in nonstretched NRVMs.

**FAK activation by stretch is dependent on RhoA activity.** We next examined the effect of C3 exoenzyme on FAK activation induced by mechanical stress in NRVMs. C3 exoenzyme selectively ADP-ribosylates small G proteins of the Rho subfamily at Asn41, thereby blocking their action (17). Although pretreatment with C3 exoenzyme had no effect on basal FAK phosphorylation, it markedly inhibited the stretch-induced FAK phosphorylation, suggesting that FAK activation by stretch is dependent on RhoA activity.

Fig. 5. RhoA antisense oligodeoxynucleotide (ODN) attenuates stretch-induced FAK and ERK1/2 activation in NRVMs. A: laser confocal analysis of control (I and II) and antisense-transfected (III and IV) NRVMs double labeled with phalloidin and RhoA antibody and cultured in medium containing serum for 18 h. B: representative anti-RhoA, -FAK, -pFAK, -ERK1/2, and -pERK1/2 immunoblots of extracts from nonstretched NRVMs transfected with sense (S) and antisense (AS) oligonucleotides compared with nontransfected cells. C: representative immunoblots of extracts from nonstretched and stretched NRVMs transfected with antisense and sense RhoA ODNs and exposed to RhoA, FAK, pFAK, ERK1/2, and pERK1/2 antibodies and average (3 experiments) results from densitometric analysis performed with pFAK and pERK1/2 antibodies normalized by total FAK and ERK1/2, respectively. *<i>P</i> < 0.05 vs. nontransfected nonstretched cells.
FAK phosphorylation at Tyr397 (Fig. 4A). Notably, inhibition of FAK phosphorylation at Tyr397 by cyclic stretch in NRVMs was accompanied by a reduction in stretch-induced ERK1/2 phosphorylation at Thr202/Tyr204.

Next, we investigated whether signaling by ROCK, a RhoA downstream serine kinase involved in the actin filament organization, mediates stretch-induced activation of FAK in NRVMs. The cells were pretreated with the selective ROCK inhibitor Y-27632 (10 μmol/l) for 1 h and then stretched for 30 min. Stretch-induced FAK phosphorylation at Tyr397 was markedly reduced after Y-27632 treatment (Fig. 4B), but no change was observed in basal FAK phosphorylation or expression compared with untreated cells. ERK1/2 phosphorylation at Thr202/Tyr204 was also markedly reduced in stretched myocytes treated with Y-27632.

Because in nonmyocyte cells RhoA/ROCK signaling has been implicated in the organization of stress fibers (17), we next examined the influence of cytochalasin D, which induces actin filament disassembly, in the stretch-induced FAK phosphorylation at Tyr397. NRVMs pretreated with cytochalasin D exhibited a marked reduction in stretch-induced FAK and ERK1/2 phosphorylation (Fig. 4C).

We further examined the role of RhoA proteins in stretch-induced FAK activation by transfecting NRVMs with RhoA antisense. The effectiveness of RhoA oligonucleotide antisense was demonstrated by its ability to specifically reduce RhoA expression in NRVMs. Immunofluorescence (Fig. 5A) and immunoblotting (Fig. 5, B and C) analysis showed that transfection with RhoA antisense markedly reduced the expression of RhoA in NRVMs but did not change the morphology or viability of NRVMs. Transfection with RhoA antisense or sense did not change FAK or ERK1/2 baseline expression and phosphorylation (Fig. 5B). The reduction of RhoA expression by RhoA antisense oligonucleotide markedly reduced the stretch-induced phosphorylation of FAK at Tyr397 and ERK1/2 at Thr202/Tyr204 (Fig. 5C).

Figure 6 summarizes the effect of transfection with FAK sense or antisense oligonucleotides on stretch-induced phosphorylation of FAK at Tyr397 and ERK1/2 at Thr202/Tyr204 in NRVMs. Transfection with FAK sense oligonucleotide did not change the stretch-induced FAK or ERK1/2 phosphorylation compared with data presented in Fig. 1B. Transfection with FAK antisense oligonucleotide significantly reduced FAK expression, abolished stretch-induced FAK phosphorylation, and markedly attenuated ERK1/2 phosphorylation.

RhoA and FAK influence stretch-induced hypertrophic gene activation. To test the role of RhoA and FAK in the stretch-mediated hypertrophic response of NRVMs in vitro, we examined the influence of transfections with RhoA or FAK antisense oligonucleotides on expression of the β-MHC transcript. RhoA or FAK sense and antisense oligonucleotides did not change baseline expression of the β-MHC transcript in nonstretched NRVMs (Fig. 7A). Cyclic stretch for 2–4 h markedly increased the amount of the β-MHC transcript expressed by NRVMs (Fig. 7B). Transfection with RhoA or FAK antisense oligonucleotides significantly reduced the stretch-induced expression of β-MHC in NRVMs, whereas transfection with sense oligonucleotides did not change the stretch-induced expression of β-MHC in NRVMs.

DISCUSSION

Here we show that stretch-induced FAK activation in NRVMs is controlled by the RhoA/ROCK signaling pathway. This finding is supported by our demonstration that 1) prevention of RhoA signaling by the selective Rho inhibitor C. botulinum C3 exoenzyme or reduction of RhoA expression by transfecting cells with RhoA oligonucleotide antisense markedly attenuated stretch-induced FAK phosphorylation at Tyr397 in NRVMs and 2) treatment of NRVMs with the ROCK pharmacological inhibitor Y-27632 markedly attenuated stretch-induced FAK phosphorylation at Tyr397. The interaction of RhoA and FAK was further supported by the demonstration that RhoA can be recovered from anti-FAK immunoprecipitates. Stretch-induced FAK phosphorylation was also reduced by cytochalasin D, suggesting that the influence of RhoA/ROCK signaling on FAK activation is dependent on the integrity of nonsarcomeric actin filaments. Additionally, it was shown that inhibition of FAK or RhoA signaling, by specific antisense oligonucleotides, attenuated the stretch-induced expression of

![Image](http://ajpheart.physiology.org/Downloadedfrom)
β-MHC mRNA in NRVMs, supporting the idea that RhoA/ROCK and FAK signaling cooperate in the control of hypertrophic gene expression in response to mechanical stress in cardiac myocytes.

RhoA/ROCK signaling influences FAK activation by cyclic stretch. Similar to FAK, RhoA has been shown to be rapidly activated by mechanical stress in cardiac myocytes (1, 20, 39). Our present data indicate that RhoA/ROCK signaling is upstream from stretch-induced FAK activation in NRVMs. Accordingly, in nonmyocyte cells, RhoA activation increases, whereas its inhibition attenuates, FAK phosphorylation (8). This also agrees with previous data indicating that FAK mediates the effects of RhoA signaling triggered by constitutively active RhoA or activation of G protein-coupled receptors in cardiac myocytes (16, 43). Given that the RhoA/ROCK signaling pathway regulates the assembly of stress fibers (17), it is conceivable that the marked reductions of stretch-induced FAK phosphorylation in NRVMs treated with RhoA/ROCK inhibitors or cytochalasin D are related to a common impairment in the assembly of actin filaments. This implies that stretch- or agonist-induced FAK phosphorylation in NRVMs is dependent on the organization and, presumably, contractility of actin stress fibers. Although NRVMs lack bona fide actin stress fibers, they possess a highly developed actin cytoskeletal meshwork closely related to sarcomeres, organelles, and sarcotica (6, 30, 32); therefore, it is possible that, on mechanical stress, actin reorganization would drive FAK recruitment at specific subcellular sites. This idea is supported by data (8) indicating that actin filaments play an essential role in the recruitment of FAK and other signaling molecules to specific subcellular structures and also by our previous demonstration that FAK phosphorylation is accompanied by its clustering at subcellular structures such as myofilaments, Z disks, and costameres (12, 39). Further studies are necessary to determine how mechanical stress and RhoA/ROCK signaling promote the remodeling of actin filaments and contribute to FAK phosphorylation and relocation in cardiac myocytes.

Anti-RhoA staining was found to be intercalated with actin staining in nonstretched and stretched NRVMs, suggesting that RhoA is localized at the sarcomeric A bands. In stretched cells, anti-RhoA staining was also found in the area of the cell nucleus, indicating that RhoA might be located at nuclear or perinuclear sites in stretched NRVMs. Immunoblotting analysis of NRVM fractions showed that RhoA could not be detected in the nuclear fraction, indicating that RhoA is probably located at perinuclear structures in stretched cells. These data indicate that the location of RhoA in stretched NRVMs contrasts to the previously demonstrated location of FAK, in that RhoA staining seems to show no major change in its location in stretched compared with nonstretched cells. This indicates that activation by mechanical stress is not accompanied by major changes in the distribution of RhoA in cardiac myocytes.

**Fig. 7.** RhoA and FAK antisense ODNs block stretch-induced β-myosin heavy chain (β-MHC) mRNA expression in NRVMs. A: RT-PCR of β-MHC and β-actin mRNA in nonstretched NRVMs transfected with RhoA or FAK sense or antisense ODNs. B: RT-PCR of β-MHC and β-actin mRNA in nonstretched and stretched NRVMs transfected with RhoA sense or antisense ODNs and average (3 experiments) results of densitometric analysis of β-MHC normalized by β-actin. *P < 0.05 vs. nontransfected nonstretched cells.
This assumption is further supported by our demonstration that cyclic stretch reduces the amount of RhoA in the precipitates of FAK antibody. This also agrees with data from a previous study (18) indicating that, on activation, RhoA, as well as ROCK, is recruited to local actin stress fibers (18). In contrast, a recent study (20) showed that a significant fraction of RhoA is localized in purified caveolae and that stretch results in the dissociation of RhoA from the caveolar fraction of NRVMs. Differences in the experimental approach, i.e., immunostaining vs. biochemical separation, and the relatively low resolution of immunofluorescence to detect minor displacement of proteins in cells might explain the apparent discrepancy in the results.

**Regulation of stretch-induced hypertrophic genes by RhoA/FAK signaling.** We have shown that RhoA signaling plays a critical role in stretch-induced expression of β-MHC. In addition, the demonstration that inhibition of RhoA/ROCK signaling markedly attenuated stretch-induced FAK activation and that FAK antisense oligonucleotide induced a comparable reduction of stretch-induced expression of β-MHC supports the idea that FAK mediates the influence of RhoA on stretch-induced gene expression in NRVMs. Previous studies showed that RhoA (1, 20, 25, 43) and FAK (22, 38, 39) control expression of the fetal gene atrial natriuretic factor in cardiac myocytes in response to hypertrophic agonists and mechanical stretch. Collectively, these data are consistent with a critical role for RhoA/ROCK and FAK signaling in promoting expression of the hypertrophic genetic program by mechanical stress in cardiac myocytes.

The present results also outline a pathway involving RhoA/ROCK and FAK as upstream mediators of stretch-induced ERK1/2 phosphorylation. Additionally, we have demonstrated that the mechanisms implied in stretch-induced ERK1/2 activation do not depend on AT1 receptor activation. This suggests that the activation of this pathway by mechanical stress is not dependent on autocrine/paracrine mechanisms mediated by ANG II. RhoA (1, 10, 25, 44) and FAK (9, 13) signaling have been implicated in the regulation of ERK1/2 and other MAP kinases. However, the role of RhoA signaling in mediating stretch-induced ERK1/2 activation in cardiac myocytes is controversial. It has been shown (20) that inhibition of RhoA signaling by disruption of its proper location on caveolae does not prevent ERK1/2 phosphorylation by steady stretch. Similar to our present data, it was recently demonstrated (27) that RhoA is an important regulator in mediating the phosphorylation of ERK1/2 by cyclic stretch in NRVMs. Thus discrepancies in the results concerning the role of RhoA signaling on stretch-induced ERK1/2 activation might be dependent on differences in signal transduction pathways elicited by steady vs. cyclic stretch.

ERK1/2 have been implicated as important transducers of the hypertrophic growth response in cell culture-based studies and in the intact heart (14, 31, 35, 36). Our present data suggest a role for ERK1/2 in mediating the effects of RhoA/ROCK and FAK signaling pathways in regulation of the stretch-induced expression of β-MHC in NRVMs. One potential mechanism whereby this signaling pathway promotes hypertrophy is by regulating activation of GATA4, a cardiac-expressed transcription factor, which has been demonstrated to be involved in cardiac hypertrophy and is directly regulated by ERK1/2 and p38 MAP kinase signaling effectors (3). A linkage between Rho/ROCK and ERK1/2/GATA-4 pathways has been shown in myocardial cell hypertrophy (44). However, the present results do not exclude the possibility that multiple downstream effectors are involved in the influence of FAK on early gene regulation in response to mechanical stress.

In conclusion, the present report demonstrates that RhoA/ROCK signaling plays a critical role in FAK activation by mechanical stress in cardiac myocytes. Furthermore, the demonstration here that RhoA and FAK signaling control the stretch-induced expression of β-MHC in NRVMs indicates that RhoA and FAK are key elements in the regulation of the hypertrophic genetic program in cardiac myocytes in response to mechanical stress. The relative importance of these mechanisms in phenotypic myocardial changes, such as hypertrophy and heart failure, needs further investigation.

**REFERENCES**


