Activation of focal adhesion kinase by protein kinase Cε in neonatal rat ventricular myocytes

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FOCAL ADHESIONS link the extracellular matrix to the actin cytoskeleton through integrins within the sarcolemma. Focal adhesion kinase (FAK) is a nonreceptor protein tyrosine kinase that is an important constituent of the focal adhesion complex. FAK undergoes activation and tyrosine autophosphorylation at a specific residue (Y397) after integrin engagement and stimulation by growth factors in neonatal rat ventricular myocyte (NRVM) cultures (13, 30, 41). In addition to performing a structural role in cell adhesion, this complex provides a site for bidirectional signal transduction through the activation of local signaling molecules during cardiomyocyte hypertrophy (12, 14, 20, 23, 30). We (13) have previously shown that FAK is necessary for sarcomeric assembly during endothelin (ET)-induced NRVM hypertrophy. In addition, we demonstrated that disruption of focal adhesions by adenoviral (Adv)-mediated overexpression of the COOH-terminal region of FAK (FRNK), inhibited FAK-dependent signaling and induced adhesion-dependent apoptosis or anoikis (16).

We and others (10, 11, 13, 24, 43) have shown that adhesion- and growth factor-dependent FAK signaling may require upstream activation of one or more isoenzymes of protein kinase C (PKC). However, cardiomyocytes express several PKC isoenzymes, two of which, PKCε and PKCδ, are activated after ET stimulation (7, 33). Therefore, we have utilized replication-defective Adv that encodes mutant forms of PKCε and PKCδ to examine which isoenzymes are involved in FAK activation and phosphorylation at Y397 (Y397pFAK). In addition, we have used pharmacological inhibitors to further interrogate the mechanism(s) by which ET and PKCs activate FAK. Data are presented to indicate that ET stimulates a complex signaling pathway, which ultimately produces actin filament assembly that is critical for FAK activation and sarcomeric assembly during cardiomyocyte hypertrophy.

MATERIALS AND METHODS

Reagents. PC-1 tissue culture medium was purchased from BioWhittaker (Walkersville, MD). Medium 199 (M199), Ca2+-free and Mg2+-free Hanks’ balanced salt solution, acid-soluble calfskin collagen, and antibiotic/antimycotic solutions were obtained from Sigma (St. Louis, MO). DMEM was obtained from Gibco-BRL (Grand Island, NY), type II collagenase from Worthington (Lakewood, NJ), and penicillin-streptomycin from Fisher Scientific/MediaTech (Itasca, IL).

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The pharmacological inhibitors Y-27632, ML-7, PP2, PP3, and cytochalasin D were obtained from Calbiochem (La Jolla, CA). Anti-FAK rabbit pAb, phospho-specific colchin pAb, and anti-collin pAb were obtained from Upstate (Lake Placid, NY). Phosphospecific Y397, Y576, Y577, Y861, and Y925 pFAK rabbit pAbS were purchased from Biosource (Camarillo, CA). Anti-PKCε mouse mAb was purchased from Transduction Laboratories (Lexington, KY). Phospho-specific ERK pAb was from Promega (Madison, WI) and anti-ERK1/2 rabbit pAb was obtained from Santa Cruz Biochemical (Santa Cruz, CA). Goat anti-rabbit and goat anti-mouse secondary Abs were obtained from Molecular Probes (Eugene, OR). All other reagents were of the highest grade commercially available and were obtained from Sigma and Baxter S/P (McGaw Park, IL).

Cell culture. Animals used in these experiments were handled in accordance with the “Guiding Principles in the Care and Use of Animals,” approved by the Council of the American Physiological Society. Ventricular myocytes were isolated from the hearts of 2-day-old Sprague-Dawley rats by collagenase digestion, as previously described (37). Myocytes were pelleted in serum-free PC-1 medium to reduce nonmyocyte contamination. The nonadherent NRVM were then plated at a density of 1,600 cells/mm² onto collagen-coated chamber slides or 35-mm-thick dishes and left undisturbed in a 5% CO₂ incubator for 14 h. Coated chamber slides or 35-mm-thick dishes and left undis-
terred in a 5% CO₂ incubator for 14–18 h. Untactched cells were removed by aspiration and washed twice in Hanks’ balanced salt solution, and the attached cells were main-
tained in a solution of DMEM/M199 (4:1) containing antibiotic/antimycotic solution. Cardiomyocytes were infected (60 min, 25°C with gentle agitation) with replication-defective Adv diluted in DMEM/M199. The medium was then replaced with virus-free DMEM/M199, and the cells were cultured for an additional 8–48 h.

Adv constructs. Replication-defective Adv encoding constitutively active (ca) rat PKCs (Adv-caPKCs) and cPKCs were constructed as previously described (17, 40). Dominant negative (dn) PKCε Adv was kindly provided by Dr. Peipei Ping of the University of Louisville Medical School (Louisville, KY) and constructed as previously described (31). Proline-rich tyrosine kinase 2 (PYK2) cDNA was kindly provided by Dr. Tom Parsons of the University of Virginia, and a replication-decorative Adv encoding the green fluorescent protein (GFP)-tagged, wild-type (wt) kinase was constructed using the Adeno-X Expression System from BD Biosciences Clontech (Palo Alto, CA) (16). To control for Adv infection, replication-defective Adv encoding cytoplasmic (cyto) (13) or nuclear-encoded (ne) (17) β-galactosidase (βgal) or GFP (16) were used. The multiplicity of viral infection (MOI) was determined by viral dilution assay in human embryonic kidney-293 cells grown in 96-well clusters.

Immunolocalization. NRVM grown on chamber slides were fixed and permeabilized (16). Myocytes were stained with a polyclonal antibody specific for FAK and a monoclonal antibody that recognizes PKCe. Appropriate FITC- or rhodamine-conjugated secondary antibodies were used to visualize the proteins of interest. Fluorescent-labeled cells were then viewed with the use of a laser scanning confocal microscope (model LSM 510, Zeiss).

Western blot analysis. NRVM were washed once in ice-cold PBS and homogenized in lysis buffer containing 1% Triton X-100 with protease inhibitors (38). Equal amounts of extracted cellular proteins were separated on 7.5% SDS-polyacrylamide gels with 4% stacking gels. Proteins were transferred to polyvinylidene difluoride membranes with the use of the recommended trans-
fer buffer. Western blots were probed with antibodies specific for FAK, the phosphorylated forms of FAK at Y397, Y576, Y577, Y861, and Y925, ERK1/2, colfin, or the phosphorylated forms of ERK1/2 or colfin. Primary antibody binding was detected with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and visualized by enhanced chemiluminescence (Amersham; Arlington Heights, IL). Band intensity was quantified with the use of laser densitometry.

Data analysis. Results were expressed as means ± SE. Normality was assessed with the use of the Kolmogorov-Smirnov test, and homogeneity of variance was assessed with the use of Levene’s test. Data were compared by one-way blocked ANOVA, followed by the Student-Newman-Keuls test. Differences among means were considered significant at P < 0.05. Data were analyzed with the use of SigmaStat statistical software (version 1.0; Jandel Scientific; San Rafael, CA).

RESULTS

ET increases Y397pFAK through mechanism involving PKCs. Initial experiments were performed to ex-
amine the effect of ET on Y397pFAK. As seen in Fig. 1A, FAK was highly phosphorylated at Y397 under basal conditions. ET stimulation (10 nmol/l, 2–10 min) resulted in a further twofold increase in the level Y397pFAK, which peaked at 10 min and remained elevated for up to 30 min. The results of four experi-
ments are summarized in Fig. 1B.

To investigate the PKC dependence of basal and ET-induced Y397pFAK, NRVM were pretreated with the nonselective PKC inhibitor chelerythrine (5 μmol/l, 1 h) and then stimulated with ET (10 nmol/l, 10 min). As seen in Fig. 1C, chelerythrine markedly reduced both basal and ET-stimulated Y397pFAK. ET-induced ERK activation was also partially dependent on PKCs, consistent with a role for PKCe in ERK activation (17, 40), although basal levels of ERK2 phosphorylation were modestly elevated after chelerythrine treatment. The results of 5–8 experiments summarizing Y397pFAK and ERK phosphorylation are shown in Fig. 1, D and E, respectively.

Overexpression of caPKCe, but not cPKCs, is sufficient to activate FAK. Previous studies have shown that ET stimulation of NRVM induces the membrane translocation of the novel PKC isoenzyme PKCe and PKCβ, but not the calcium-dependent PKC isoenzyme PKCe (7, 33). Therefore, we generated replication-decorative Adv encoding caPKCe and cPKCs and exam-
ined their effects on Y397pFAK at multiple time points after Adv infection. Adv-neβgal was used to control for nonspecific effects of Adv infection. As shown in Fig. 2A, caPKCe overexpression increased Y397pFAK as early as 8 h postinfec-
tion, which continued to increase over the 48-h time period examined. In addition, total FAK levels (phosphorylated and un-
phosphorylated) were also significantly increased at each time point. Data summarizing the results of 7–15 experiments are depicted in Fig. 2, C and D.

In marked contrast to the effects of Adv-caPKCe, overexpression of caPKCβ resulted in a time-depen-
dent decrease in Y397pFAK and total FAK (Fig. 2B). Figure 2C summarizes the results of 5–6 experiments demonstrating that Y397pFAK was decreased to 0.8 ± 0.1- and 0.6 ± 0.1-fold of the time-matched control Adv
at 24 and 48 h, respectively. Figure 2D summarizes the results of 4–5 experiments and shows that total FAK levels were decreased by 48 h to 0.6 ± 0.1-fold of time-matched control Adv.

To determine the effect of Adv infection alone on Y397pFAK and total FAK levels over time, multiple experiments were performed comparing uninfected NRVM with NRVM infected with Adv-neβgal for 8, 24, or 48 h (all cells were harvested at the same time and day). Figure 2E depicts a representative Western blot demonstrating that infection with Adv-neβgal did not substantially alter either Y397pFAK or total FAK levels over the time period examined compared with uninfected NRVM.
Overexpression of dnPKCe reduces basal and ET-induced Y397pFAK. With the use of a replication-defective Adv encoding dnPKCe, we next addressed whether PKCe was necessary for FAK activation in response to ET stimulation. Adv-cytoβgal was used to control for nonspecific effects of Adv infection. As shown in Fig. 3A, Adv-dnPKCe decreased both basal and ET-induced Y397pFAK. The results of four experiments are quantitatively assessed in Fig. 3B and demonstrate that dnPKCe overexpression reduced basal levels of Y397pFAK to 0.4 ± 0.2-fold of Adv-cytoβgal-infected cells. ET-induced FAK activation was also significantly decreased from 1.4 ± 0.1-fold in Adv-cytoβgal-infected NRVM to 0.5 ± 0.1-fold after dnPKCe overexpression. Total FAK levels also decreased somewhat after dnPKCe overexpression; however, this difference did not reach statistical significance (Fig. 3C).

PKCe and FAK colocalize to focal adhesions. Because PKCe was both necessary and sufficient to activate FAK, we next examined whether the two kinases were localized to the same region of the cardiomyocyte. We previously showed that FAK and the focal adhesion protein paxillin were colocalized to the cell-substratum
interface in control NRVM (16). As shown in Fig. 4A, double-label confocal microscopy revealed that PKCε and FAK also colocalized to basilar regions of control cells. Although PKCε was present in the same focal adhesion sites as FAK, additional PKCε staining was detected in the perinuclear region as well as within cell-cell junctions. PKCε and FAK colocalization was also apparent in cells infected with Adv-neζgal (Fig. 4B). Markedly increased levels of immuno-reactive PKCε were detected in NRVM infected with Adv-caPKCε (Fig. 4C). caPKCε overexpression resulted in the formation of filapodia-like projections in many of the cells, consistent with observations from our earlier studies (17, 40). caPKCε overexpression also increased FAK staining, thus confirming the results of the Western blot experiments depicted in Fig. 2. Intense regions of FAK and PKCε staining were noted in a banded pattern, consistent with the appearance of costameres along the lengths of the elongated cell projections. These projections terminated in FAK- (Fig. 4C) and paxillin-positive (40) focal adhesions. Thus PKCε and FAK colocalize to focal adhesions and costameres in control and caPKCε-overexpressing NRVM.

Inhibition of Rho kinase abrogates ET- and PKCε-induced activation of Y397pFAK and coflin. Rho belongs to a family of small GTPases that regulate actin stress fiber formation, focal adhesion assembly, and cellular contraction in response to cell adhesion and growth factors in multiple cell types (35, 36). A downstream target of GTP-bound Rho is p160ROKβ [Rho-associated coiled-coil-containing protein kinase (ROCK)]. ROCK, in turn, indirectly phosphorylates the actin-binding protein coflin via activation of LIM kinase, directly phosphorylates and inactivates myosin light chain (MLC) phosphatase, and directly phosphorylates MLC2 leading to nonsarcomeric myosin-dependent cell contraction (1, 21). ET has been shown to promote significant activation of RhoA in neonatal myocytes (8). We therefore investigated ROCK as a potential intermediary in ET- and/or PKCε-induced activation of Y397pFAK and coflin. NRVM were pretreated with the selective ROCK inhibitor Y-27632 (10 μmol/l, 1 h) and then treated with ET (10 nmol/l, 10 min) or infected with Adv-neζgal or Adv-caPKCε in the presence of Y-27632. Figure 5A demonstrates that both basal and ET-induced Y397pFAK were markedly reduced with Y-27632 treatment. In addition, there was a threefold increase in ET-induced phosphorylation of coflin. Interestingly, inhibition of ROCK completely abrogated both basal and ET-induced coflin phosphorylation. The results of 4–8 experiments summarizing Y397pFAK and coflin phosphorylation are shown in Fig. 5, B and C, respectively. Figure 5E demonstrates that ET-induced Y397pFAK and coflin phosphorylation in NRVMs overexpressing neζgal and caPKCε. In addition, overexpression of caPKCε, like ET treatment, increased coflin phosphorylation threefold. Figure 5, F–H, represents 3–4 experiments summarizing co-

![Fig. 3. Overexpression of dominant-negative (dn)PKCε reduces basal and ET-induced Y397pFAK. A: NRVM were infected with either Adv-cytoζgal or Adv-dnPKCε (750 MOI, 72 h) and then treated with ET (10 nmol/l, 10 min). Western blots (50 μg of extracted protein) were probed with either an antibody specific for the Y397pFAK site or an antibody that recognizes both the phosphorylated and unphosphorylated forms of FAK. The position of molecular weight markers is indicated to the right of the blot. B and C: quantitative analysis of four Western blot experiments. The levels of Y397pFAK and FAK are shown normalized to those observed after Adv-cytoζgal infection. Data are means ± SE. *P < 0.05 vs. Adv-cytoζgal.](http://ajpheart.physiology.org/)

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filin phosphorylation, Y397pFAK, and FAK, respectively.

We have previously shown that ET-induced ERK activation is independent of Y397pFAK in NRVM overexpressing FRNK, the autonomously expressed, COOH-terminal domain of FAK (16). Therefore, we examined ERK activation as a measure of normal cardiomyocyte responsiveness after Y-27632 treatment. As seen in Fig. 5A, ET-induced ERK activation remained unaffected by ROCK inhibition, although basal ERK activation was diminished. The results of four experiments summarizing ERK phosphorylation is shown in Fig. 5D. Similarly, in NRVM overexpressing neβgal and caPKCε, inhibition of ROCK did not substantially alter ERK phosphorylation.

To examine the role of myosin phosphorylation in FAK activation, NRVM were pretreated with an inhibitor of MLC kinase, ML-7 (3 μmol/l, 1 h), in the presence or absence of ET (10 nmol/l, 10 min). We found that ML-7 treatment had no effect on either basal or ET-induced Y397pFAK, or ERK phosphorylation. In addition, disruption of myosin/actin cross-bridge forma-

Fig. 4. PKCε and PAK colocalize to focal adhesions. NRVMs were maintained in control medium (A), infected with Adv-neβgal (25 MOI, 48 h) (B), or infected with Adv-caPKCε (25 MOI, 48 h) (C). Cells were fixed and double labeled with an anti-PKCe mAb and an anti-FAK pAb. Rhodamine-conjugated goat-anti-mouse IgG (red; PKCe) and FITC-conjugated goat-anti-rabbit IgG (green; FAK) were used for visualization by laser confocal microscopy (1 μm optical sections obtained at the cell-substratum interface). All images were taken with identical laser and microscope settings. Areas of colocalization appear as yellow.
tion with BDM (7.5 mmol/l, 1 h) also did not affect FAK phosphorylation. Furthermore, neither inhibition of calcium transients with nifedipine (10 μmol/l, 1 h) nor chelation of intracellular calcium with BAPTA-AM (50 mmol/l, 0.5-h pretreatment, 0.5-h washout) altered the phosphorylation of FAK (data not shown).

Depolymerizing actin filaments reduces ET-induced phosphorylation of cofilin and Y397pFAK. Because FAK activation was coincident with the phosphorylation of cofilin, and cofilin phosphorylation regulates F-actin assembly (27), we induced actin filament disassembly with cytochalasin D (1 μmol/l, 1 h) and examined its effects on both ET-induced phosphorylation of cofilin and Y397pFAK. As shown in Fig. 6, NRVM pretreated with cytochalasin D exhibited a marked reduction in both basal and ET-induced cofilin and FAK phosphorylation, with no significant change in ERK phosphorylation. In addition, the NRVMs were infected with Adv-neβgal or Adv-cPKCε (25 MOI, 48 h) in the presence of cytochalasin D (1 μmol/l, 48 h). Western blots (50 μg of extracted protein) were probed with the following antibodies: phosphorylated cofilin (pCo), cofilin (both phosphorylated and unphosphorylated forms), phosphorylated ERK1/2 (pERK1/2), and an antibody that recognizes both the phosphorylated and unphosphorylated forms of ERK1/2. The numbers at the right of each blot indicate the position of molecular weight markers. B–D and F–H: quantitative analysis of 3–4 Western blot experiments. Y397pFAK/FAK, pERK, and pCo/Co or Y397pFAK, FAK, and pCo/Co levels are shown normalized to those observed without Y-27632, ET, or caPKCε treatment. Data are means ± SE. *P < 0.05 vs. `untreated, †ET or caPKCε treated, or #Y-27632 treated, respectively.

Overexpression of PYK2 does not alter Y397pFAK. Ping and co-workers (32) have demonstrated that PKCε forms a signaling module with multiple structural and signaling proteins in cardiomyocytes. One signaling molecule that physically associates with PKCε is PYK2, a protein closely related to FAK (32).
We have shown that PYK2 is expressed in both neonatal and adult rat ventricular myocytes and is phosphorylated in response to ET (3). We have also demonstrated that inhibition of Y397pFAK by GFP-FRK overexpression abrogates ET-induced phosphorylation of PYK2 at Y402, its putative autoactivation site (16). Therefore, to address whether the activation of PYK2 affects FAK activation, we infected NRVM with Adv-GFP or Adv-GFP-wtPYK2 (1 MOI, 8–48 h) and examined their effects on FAK levels and Y397pFAK. By 8 h after Adv-GFP-wtPYK2 infection, an abundant amount of the exogenous protein was detected, which continued to increase over the 48-h time period examined. However, overexpression of GFP-wtPYK2 did not alter either Y397pFAK or total FAK levels over the time period examined (data not shown).

Inhibition of Src does not alter Y397pFAK, but decreases Y576pFAK, Y577pFAK, Y861pFAK, and Y925pFAK. PKCε also forms a signaling module with Src and other Src-family nonreceptor protein tyrosine kinases in cardiomyocytes (32), and PKCε phosphorylates and activates Src (42). In other cell types, FAK activation and autophosphorylation at Y397 provides a docking site for Src, which, in turn, phosphorylates FAK at other sites. We therefore examined whether ET-induced FAK phosphorylation at Y397 and other sites (Y576, Y577, Y861, and Y925) were dependent on the upstream activation of Src. As seen in Fig. 8A, NRVM stimulated with ET exhibited increased levels of Y397pFAK, Y576pFAK, Y577pFAK, Y861pFAK, and Y925pFAK. Treatment with the highly specific, Src-family kinase inhibitor PP2 (50 μmol/l, 1 h) decreased both basal and ET-induced phosphorylation at all sites except for Y397 compared with untreated or PP3-treated NRVM. Figure 8, B–G, summarizes the results of 5–7 experiments. Similar results were obtained in cells that overexpressed caPKCε (data not shown).

DISCUSSION

Inside-out signaling pathways initiated by Gq-coupled receptors result in FAK-dependent sarcomeric assembly (13). Agonists that activate Gq-coupled receptors increase cell spreading and formation of sarcomeres at the cell periphery, a process that requires the formation of premyofibrils (2). Premyofibrils, in turn,
contain actin filaments and Z-bodies, which are precursors to Z-bands and resemble focal adhesions in protein composition (9, 34). Premyo filibrils appear at the spreading edge of embryonic chick cardiomyocytes and develop into mature myo filibrils when several Z-bodies fuse into a single Z-band. Interestingly, nonmuscle myosin IIB is found between Z-bodies in premyo filibrils; however, only mature myo filibrils contain muscle-specific myosin II. To a large extent, however, many of the signaling molecules involved in the regulation of premyo fibril formation are not known. In the present study, we describe a pathway involving PKC/H9280, ROCK, coflin, and actin filament assembly, which is necessary for ET-induced FAK activation and subsequent sarcomeric assembly.

Signaling through PKCs was required for Y397pFAK because the PKC inhibitor chelerythrine abrogated ET-induced FAK activation. In addition, we found that basal levels of Y397pFAK were similarly reduced by PKC inhibition, suggesting that the maintenance of signaling through focal adhesions requires PKCs. We have previously demonstrated that ET-induced FAK tyrosine phosphorylation was inhibited by chelerythrine in NRVM (13). Similarly, in other cell types, PKC inhibitors reduced FAK phosphorylation, whereas phorbol esters, which activate PKCs, increased FAK phosphorylation (10, 11, 24, 43). Despite this accumulating evidence, isoenzyme-selective PKC regulation of FAK has not yet been demonstrated in any cell type. We now show that overexpression of caPKC/H9280 increased both Y397pFAK and total FAK, whereas overexpression of dnPKC reduced basal and ET-induced Y397pFAK. In contrast, overexpression of caPKC/H9254 decreased both Y397pFAK and total FAK. These results are consistent with other data indicating that PKC/H9280 and PKC/H9254 have opposing effects on cardiomyocyte cell survival and injury (6, 17). Furthermore, caPKC/H9280 overexpression elevated both Y397pFAK and total FAK levels as early as 8 h postinfection, which coincided with maximal ERK activation (17). However, ET-induced ERK activation appeared to be independent of Y397pFAK (16). Conversely, overexpression of caPKC did not decrease FAK activation until 24 h postinfection, nor did total FAK levels decrease until 48 h postinfection. We have previously shown that NRVM overexpressing caPKC undergo apoptosis within 24–48 h after Adv infection (17) as do NRVM overexpressing FRNK (16). It is interesting to speculate that the PKC-induced decrease in Y397pFAK is the initiating signal resulting in
the apoptotic response. However, further experimentation would be necessary to test this hypothesis.

We demonstrated that PKCe and FAK colocalize to regions of the cell consistent with the appearance of costameres and focal adhesions. In addition, overexpressing caPKCe resulted in increased FAK colocalization as well as increased immunoreactive FAK. Colocalization of PKCe and FAK has been shown in vascular smooth muscle cells after binding to fibronectin (15), and we have previously identified PKCe within costameres of NRVMs (5). These results are also consistent with studies examining the function and physical interaction of receptor for activated C kinase (RACK1), with PKC isoenzymes (28). RACK1 was shown to directly interact with the cytoplasmic tail of β-integrins in vitro (26). Furthermore, overexpression of RACK1 in NIH3T3 cells increased actin stress fiber and focal adhesion formation, and induced cell spreading. In addition, FAK and paxillin tyrosine phosphorylation increased, thereby linking activated PKCs directly to integrins (18). Although RACK1 preferentially binds Ca2+-dependent PKC isoenzymes, Pass et al.
(29) have shown that transgenic mice overexpressing high levels of PKCe in ventricular myocytes have enhanced RACK1 protein expression and increased RACK1-PKCe interaction. Recently, Besson et al. (4) showed that PMA stimulation of human glioma cells increased focal adhesion formation and coimmunoprecipitation of PKCe, RACK1, and β1- and β3-integrins. Therefore our data, which demonstrate that FAK and PKCe are colocalized to focal adhesions and costameres even under basal conditions, provides additional evidence in support of a functional and structural link between the two signaling kinases.

Several investigators (2, 19, 22) have shown that Rho and ROCK are necessary and/or sufficient for myofibrillar reorganization and ANF expression after activation of Gq-coupled receptors in NRVM. We now show that inhibition of ROCK with Y-27632 reduced basal, and ET- and PKCe-induced activation of Y397pFAK, demonstrating the importance of the Rho/ROCK pathway in FAK-dependent sarcomeric assembly. We then interrogated two pathways downstream of ROCK: namely, the LIM kinase/cofilin pathway, which results in F-actin assembly, and the MLC2/MLC phosphatase pathway, which results in non-sarcomeric myosin-dependent cell contraction (1, 21). We found that phosphorylation of cofilin, which is an actin-depolymerizing factor that is inactivated on phosphorylation by LIM kinase (39), was increased threefold after ET treatment. ET-induced regulation of actin assembly through the phosphorylation of cofilin may occur via upstream activation of PKCe because cofilin phosphorylation was also increased threefold after caPKCe overexpression. We confirmed the importance of assembled actin filaments in FAK activation by depolymerizing actin filaments with cytochalasin D and demonstrated that basal, ET-, and caPKCe-induced Y397pFAK were all reduced after this treatment. Surprisingly, we found that cytochalasin D also reduced both basal and ET-induced cofilin phosphorylation, which would indicate that one or more of components of this signaling pathway requires an intact actin cytoskeleton. This requirement may be at the level of PKCe, rho, ROCK, or cofilin localization.

In contrast, we found that interference with ROCK-dependent myosin phosphorylation had no effect on either basal or ET-induced Y397pFAK. Similarly, neither inhibiting actin/myosin cross-bridge formation with BDM nor interference with intracellular Ca2+ concentration transients with nifedipine or BAPTA-AM reduced FAK phosphorylation. Although Izumo’s group (2) has shown that MLC kinase is indeed involved in ET- and angiotensin II-mediated sarcomeric organization, our results indicate that FAK and MLC kinase reside in parallel signaling pathways. Both pathways appear necessary for sarcomeric assembly during Gq-induced cardiomyocyte hypertrophy.

It should be pointed out that in many of the experiments where inhibitors or Adv-dnPKCe was used, ET treatment was still able to activate FAK somewhat. In most of these experiments, however, no statistical difference was found between the groups following quantitative analysis of the data (drug/Adv alone vs. drug/Adv with ET treatment). The only exception to this was the ability of ET to activate FAK slightly after inhibition of ROCK. Nevertheless, it is likely that additional pathways are involved in ET-induced FAK activation that do not require PKCe, ROCK, or intact actin filaments.

Finally, we evaluated the potential roles of PKCe, PYK2, and Src in ET-induced Y397pFAK. Although PKCe was clearly an upstream regulator of FAK activation, neither PYK2 nor Src were necessary for this effect. Ping et al. (32) have previously demonstrated that PKCe forms a signaling module with both PYK2 and Src in cardiomyocytes. However, this module does not appear to be functionally important for Y397pFAK. Nevertheless, it is conceivable that PKCe-dependent activation of Src is required for Src binding to FAK, and phosphorylating FAK at other sites. PYK2 may also be involved in FAK phosphorylation at other sites because PYK2 phosphorylated FAK at sites other than...
Y397, Y576, Y577, and Y925 when coexpressed in rat liver epithelial cells (25). However, Src may be an intermediate in this process, as FRNK, a dominant negative inhibitor of FAK, prevented both FAK and PYK2 autophosphorylation in NRVM (16).

In summary, we have described a multicompartment signaling pathway that links G__i__-coupled receptor activation to FAK, a critical signaling kinase involved in cell survival and sarcomeric assembly (Fig. 9). This pathway provides an important link between “outside-in” signaling via the ET_A receptor and “outside-in” signaling via integrins (12, 14, 20, 23, 30) in cardiomyocytes. Both pathways converge on FAK and are critical for the hypertrophic response.

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DISCLOSURES
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