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Differential effects of Pyk2 and FAK on the hypertrophic response of cardiac myocytes

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Abstract

The related cytoplasmic non-receptor tyrosine kinases Pyk2 (proline-rich tyrosine kinase 2) and FAK (focal adhesion kinase) have been implicated in phenylephrine-induced G-protein-coupled receptor-mediated signaling mechanisms leading to cardiomyocyte hypertrophy. We report that, in phenylephrine-stimulated neonatal rat ventricular myocytes (NRVM), Pyk2 augments expression of the hypertrophic marker atrial natriuretic factor (ANF) but reduces cytoskeletal organization and cell spreading. In contrast, FAK attenuates ANF production but does not alter cytoskeletal organization and cell spreading. Pyk2 and FAK exhibit differential localization in both unstimulated and phenylephrine-stimulated myocytes. Pyk2 catalytic activity is required for Pyk2 to augment ANF secretion but is not necessary to reduce cell spreading. Pyk2 autophosphorylation is required but not sufficient for Pyk2 to augment ANF secretion. Expression of the Pyk2 FERM domain as an autonomous fragment inhibits phenylephrine-mediated ANF secretion and reduces cell spreading. In addition, expression of the Pyk2 FERM domain inhibits the ability of Pyk2 to augment ANF secretion; this is correlated with reduced Pyk2 autophosphorylation. These data indicate that Pyk2 and FAK have different roles and occupy different positions in signaling pathways leading to the development of cardiomyocyte hypertrophy.

Keywords

Focal adhesion kinase; Proline-rich tyrosine kinase 2; Cardiomyocytes; Hypertrophy; Signaling; Rat (Sprague Dawley)

Introduction

Cardiac hypertrophy is an adaptive response of the heart to a variety of intrinsic and extrinsic stimuli including hemodynamic stress, myocardial infarction, and neurohormonal factors (Hunter and Chien 1999) and is characterized by an increase in cell mass and myofibrillar content without an increase in myocyte number. Although it is a compensatory process that leads to a heart better suited for increased workload, prolonged hypertrophy can become deleterious, resulting in cardiomyopathy, heart failure, and sudden death (Sugden

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1999). At the cellular level, hypertrophic stimuli induce distinct morphological and biochemical changes including the expression of immediate early genes, re-expression of fetal genes in ventricular myocytes, and increased expression of sarcomeric contractile proteins together with an increased assembly of organized myofibrils (Hunter and Chien 1999; Iwaki et al. 1990). Substantial evidence implicates integrins and G-coupled receptors in the cellular signaling pathways underlying the development of cardiomyocyte hypertrophy and the progression to heart failure (Brancaccio et al. 2006; D'Angelo et al. 1997). However, the cellular mechanisms that synchronize the regulation of gene expression and induce cytoskeletal organization and sarcomeric assembly in hypertrophying myocytes is not completely understood.

Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase that is a key constituent of the focal adhesion complex that couples integrin-matrix interaction to intracellular signaling events. In cardiomyoctes, FAK is rapidly activated following integrin clustering and modulates signaling pathways by functioning both as an effector kinase and as a scaffold protein (Eble et al. 2000; Franchini et al. 2000; Kuppuswamy et al. 1997). Focal adhesions form part of the costameres, which link the Z-disk to the sarcolemmal membrane. Treatment of cardiomyocytes with anti-β_1 integrin antibodies alters the organization and alignment of sarcomeres thereby establishing a correlation between focal adhesions, costameres, and the assembly of sarcomeres (Hilenski et al. 1992). Furthermore, disruption of focal adhesion complexes inhibits FAK-dependent signaling and induces adhesion-dependent apoptosis or anoikis (Heidkamp et al. 2002) substantiating a role for FAK-dependent signaling pathways in myocyte growth and survival.

Proline-rich tyrosine kinase 2 (Pyk2) is a cytoplasmic non-receptor tyrosine kinase that is closely related to FAK (Avraham et al. 1995; Sasaki et al. 1995). Similar to FAK, Pyk2 serves both as a kinase effector and a scaffold protein that mediates downstream signaling by interacting with various adapters and effectors including several that also interact with FAK (Avraham et al. 2000). In spite of their similarities, Pyk2 and FAK possess a number of significant differences including tissue distribution, intracellular localization, and modes of activation (Avraham et al. 2000; Bayer et al. 2001; Eble et al. 2000). Although Pyk2 expression and phosphorylation have been demonstrated in cardiomyocytes (Bayer et al. 2001, 2003), their role in cardiomyocytes remain largely undefined.

Together, the FAKs are uniquely positioned to transduce information from interactions with the extracellular matrix and soluble mediators through cell surface integrins, receptor tyrosine kinases, and G-protein-coupled receptors into the activation of intracellular signaling pathways that modulate cell growth. In the present study, we have investigated the roles of Pyk2 and FAK in the hypertrophic response of adrenergically stimulated neonatal rat ventricular myocytes (NRVM). This in vitro model has been widely utilized to study hypertrophic signaling events in cardiac myocytes (Clerk and Sugden 1999; McDonough et al. 1993; Simpson 1983). We report that Pyk2 expression and FAK expression differentially regulate phenylephrine-induced ANF secretion and cell spreading and exhibit differential distribution in response to phenylephrine stimulation. Furthermore, the FERM domain of Pyk2 plays a central role in mediating the effects of Pyk2. These results suggest that Pyk2 and FAK occupy different positions in cellular signaling pathways and have distinctly different contributions to the temporal development of the adrenergic-mediated hypertrophic response.

Materials and methods

Antibodies and reagents

Affinity-purified antibody to the HA epitope tag was obtained from Upstate Biotechnology (Lake Placid, N.Y.). Anti-FLAG M2 monoclonal antibody was obtained from Sigma (St. Louis, Mo.). Site- and phospho-specific polyclonal antibodies to FAK pY397 and Pyk2 pY402 were obtained from Biosource International (Camarillo, Calif.). Fluorescein isothiocyanate (FITC)- and Cy5-conjugated secondary antibodies were obtained from Jackson Research Laboratories (West Grove, Pa.). Rhodamine phalloidin was obtained from Molecular Probes (Eugene, Ore.). Fibronectin-coated plates and glass slides were obtained from Fisher Scientific (Houston, Tex.).

Isolation and culture of rat ventricular myocytes

Neonatal rat ventricular myocytes were isolated and prepared as previously described (Ross et al. 1998). Briefly, myocytes were dispersed from ventricles of 1- to 3-day-old Sprague-Dawley rats by digestion with collagenase II and pancreatin at 37°C. The cell suspension was separated on a discontinuous Percoll gradient to obtain myocardial cell cultures with >95% myocytes. The myocytes were pre-plated in 10-cm culture dishes in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum for 1–2 h to remove non-myocyte cells. The unattached myocytes were removed, and equal numbers of cells were plated on fibronectin-coated plates and maintained in the native state or were infected with recombinant adenoviral constructs as noted below. Cell cultures were maintained in serum-free medium with or without 100 μM phenylephrine for an additional 48 h at 37°C. The extent of myocyte spreading was analyzed by using a BioRad Fluor-S MultiImager work station (Bio-Rad Laboratories, Hercules, Calif.). The surface area of spread cells within representative fields was quantitated with the volume contour tool of the Quantity One Image Analysis software package v4.2. Data are presented as the percentage of the available surface area covered by the spread cells. All animal handling conformed to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996).

Measurement of ANF production

ANF secretion from NRVM into media was quantitated by competitive enzyme-linked immunosorbent assay (ELISA) as directed by the supplier (Peninsula Labs, San Carlos, Calif.).

Adenoviral expression constructs

Recombinant adenovirus encoding HA-epitope-tagged FAK was as previously described (Lipinski et al. 2003). To generate FLAG-epitope-tagged Pyk2, the coding sequence for Pyk2 was ligated downstream of a 3X FLAG epitope in pcDNA3 (Invitrogen, Carlsbad, Calif.). The Pyk2 phosphorylation-deficient mutant Y402F was generated by using the Quickchange site-directed mutagenesis system (Stratagene, La Jolla, Calif.). To generate the Pyk2 N-terminal-deletion mutant Pyk2Δ376, a fragment encoding Pyk2 residues S376-E1009 was amplified by the polymerase chain reaction (PCR) and ligated in-frame downstream of the 3X FLAG epitope in pcDNA3. The Pyk2 FERM domain, encoding Pyk2 residues R39-A367, was amplified by PCR and ligated in-frame downstream of a 3X HA epitope in pcDNA3. Recombinant E1-deleted adenoviruses were prepared by using the Ad-Easy system as described (Lipinski et al. 2003). Viruses were clonally isolated and titered. Cells were infected at matched multiplicity of infection (MOI).

Immunoprecipitation and immunoblotting

Cell lysates for Western blotting or immunoprecipitation were prepared as previously described (Pham et al. 2000). The protein content of the lysate was determined by using the BCA assay (Pierce, Rockford, Ill.). Cell lysates (150 μg) were immunoprecipitated by utilizing μMACS anti-HA microbeads as directed by the supplier (Miltenyi Biotec, Auburn, Calif.). Immunoprecipitates or whole cell lysates (5–10 μg) were resolved on 8%–16% gradient SDS-polyacrylamide gels (Novex, Carlsbad, Calif.) and transferred to nitrocellulose. Immunoblotting of the transferred proteins was performed for 1 h at room temperature and visualized by enhanced chemiluminescence (Perkin Elmer Life Sciences, Boston, Mass.).

Immunofluorescence

NRVM cells plated onto fibronectin-coated glass slides were cultured in serum-free medium or in serum-free medium containing 100 μM phenylephrine for 48 h, washed in phosphate-buffered saline (PBS), and fixed with freshly prepared 2% paraformaldehyde in PBS for 15 min at room temperature. The cells were then washed in PBS, permeabilized in 0.2% Triton X-100, washed again with PBS, and pre-blocked with PBS containing 3% BSA and 1% porcine serum for 1 h. Subsequently, cells were incubated with primary antibody in blocking buffer for 1 h at room temperature, washed, incubated with either FITC- or Cy5-conjugated secondary antibody for 1 h at room temperature, and washed three times with PBS. Slowfade Lightantifade (Molecular Probes) solutions were added to conserve fluorochromes, and coverslips were applied. Specimens were examined by using a LSM510 laser scanning confocal microscope (Carl Zeiss, Thornwood, N.Y.).

Statistics

ANF secretion data was imported into GraphPad Prism 4.0 (GraphPad Software, San Diego, Calif.). Preliminary distributional assumptions of normality were tested by using the Kolmogorov-Smirnov test. Independent-sample *t*-tests were used for analysis involving two samples, and one-way analysis of variance followed by the Dunnett post-test for tests involving more than two samples. All tests are two-tailed. Data are presented as mean±SE. Differences were considered to be significant at $P<0.05$.

Results

Pyk2 expression augments expression of hypertrophic marker ANF and reduces cell spreading

Recent studies have suggested that the non-receptor tyrosine kinase Pyk2 may be an important effector in cellular signaling pathways of cardiac hypertrophy (Melendez et al. 2002). To examine further the role of FAK signaling in cardiac hypertrophy, we have utilized a cultured NRVM model system. Adrenergic stimulation of primary cultures of NRVM induces a hypertrophic response biochemically and morphologically similar to that which occurs in the intact heart (Simpson et al. 1982). When stimulated by phenylephrine, these cells show an increase in protein synthesis and induction of expression of various biochemical markers of hypertrophy including ANF and MLC-2v (Knowlton et al. 1991; McDonough et al. 1993). In addition, we have previously demonstrated that adrenergic stimulation of NRVM significantly increases cell volume and area and relative protein synthesis (Ross et al. 1998).

To examine hypertrophic marker gene expression, NRVM were infected with increasing MOI of recombinant adenovirus encoding Pyk2 and cultured in serum-free media or in serum-free media containing 100 μM phenylephrine, and the production of the ANF was

quantitated by ELISA. Consistent with previous studies, phenylephrine stimulation of NRVM induced a robust increase in ANF production relative to NRVM maintained in serum-free media (Fig. 1a). Pyk2 expression did not alter ANF production in NRVM maintained in serum-free media (data not shown). Expression of Pyk2 in phenylephrine-stimulated myocytes resulted in a significant dose-dependent augmentation of ANF secretion relative to phenylephrine-stimulated NRVM expressing the control protein β-galactosidase (β-gal; Fig. 1a). Immunoblot analysis of NRVM cell lysates demonstrated that increased Pyk2 expression was accompanied by an increase in Pyk2 autophosphorylation as assessed with the site-specific anti-Pyk2 pY402 antibody. This finding was consistent with other studies demonstrating that the overexpression of Pyk2 leads to increased tyrosine phosphorylation (Kohno et al. 2008; Lipinski et al. 2006; Park et al. 2004). Since increased cell spreading, cytoskeletal organization, and sarcomeric assembly characterize myocyte hypertrophy (McKinsey and Olson 1999), we also examined the effect of Pyk2 expression on cell morphology. Phenylephrine stimulation of control NRVM expressing β-gal (Fig. 1c) increased the area that the cells spread relative to control NRVM (Fig. 1b) in the absence of phenylephrine stimulation (58.8% of surface area vs. 35.3%, respectively). In contrast, phenylephrine stimulation of NRVM expressing Pyk2 (Fig. 1d) reduced their spreading relative to stimulated control NRVM (44.3% of surface area vs. 58.8%). Pyk2-expressing myocytes were reduced in size relative to phenylephrine-stimulated control infected cells and possessed an irregular spindle-like morphology. These results indicated the differential effect of Pyk2 on ANF secretion and cell spreading in phenylephrine-stimulated cardiomyocytes.

Effect of FAK expression on ANF secretion and cell morphology

A role for FAK has been reported both in hypertrophic growth and survival of cardiomyocytes (Kovacic-Milivojevic et al. 2001; Melendez et al. 2002, 2004). Since Pyk2 and FAK share a high degree of homology, we compared the role of FAK with that of Pyk2 on the expression of ANF and cell spreading. Expression of FAK in non-stimulated NRVM cells did not alter ANF production relative to non-stimulated control cells (data not shown). In contrast, secretion of ANF in phenylephrine-stimulated myocytes decreased with increasing expression of FAK (Fig. 2a). Increased expression of exogenous FAK results in increased tyrosine phosphorylation of FAK (Dunty and Schaller 2002; Sieg et al. 2000). Immunoblot analysis of NRVM cell lysates indicated that increased expression of FAK was accompanied by an increase in FAK phosphorylation as indicated by increased reactivity with the anti-FAK pY397 antibody. Since expression of Pyk2 and FAK had opposite effects on phenylephrine-stimulated ANF secretion, we also examined the effect of FAK expression on cell spreading. Increased expression of FAK in phenylephrine-stimulated myocytes (Fig. 2d) increased the area that cells spread relative to control-stimulated myocytes (Fig. 2c; 78.7% vs. 62.5%) but did not produce any significant morphological alterations compared with phenylephrine-stimulated control cells. These results further suggested differential effects of Pyk2 and FAK in the hypertrophic response.

Localization of Pyk2 and FAK in response to phenylephrine stimulation

To investigate potential mechanisms by which Pyk2 and FAK differentially regulated ANF production and cell spreading, we examined, by immunofluorescence, the effect of phenylephrine stimulation on the subcellular distribution of Pyk2 and FAK. NRVM plated on fibronectin-coated glass slides were infected with recombinant adenoviruses encoding the control β-gal (Fig. 3a, b), FAK (Fig. 3c, d), or Pyk2 (Fig. 3e, f), fixed, stained, and examined by confocal microscopy. Phenylephrine stimulation of cardiomyocytes expressing the control β-gal exhibited an increase in ANF staining and cytoskeletal organization relative to non-stimulated control cells. In non-stimulated NRVM, FAK was predominantly localized in the perinuclear region, with lower immunostaining at the cell periphery (Fig. 3c).

Following phenylephrine stimulation, FAK staining increased at the cell periphery concomitant with reduced perinuclear staining (Fig. 3d) and was accompanied by a noticeable increase in myofibrillar and cytoskeletal organization. Notably, phenylephrine-stimulated FAK-expressing myocytes lacked detectable ANF staining (Fig. 3d′). In contrast, Pyk2 immunostaining was localized mainly at the cell periphery in non-stimulated NRVM (Fig. 3e) and enriched in the perinuclear area of phenylephrine-stimulated myocytes (Fig. 3f). Moreover, phenylephrine stimulation of Pyk2-expressing myocytes resulted in a significant increase in ANF staining (Fig. 3f′). In contrast to the increased cytoskeletal organization and sarcomeric structures observed in the control or the FAK-expressing myocytes (Fig. 3d″), increased expression of Pyk2 also led to a loss of well-defined myofibrillar structures at the cell periphery (Fig. 3f″). The data indicated that Pyk2 and FAK had distinct effects on the expression of the hypertrophic marker ANF and on cell morphology. Moreover, their differential localization suggested that Pyk2 and FAK probably required distinct binding partners or signaling effectors to mediate their dissimilar effects on phenylephrine-induced myocyte hypertrophy.

Autophosphorylation of Pyk2 is required but not sufficient for the capacity of Pyk2 to augment ANF secretion and to reduce cell spreading

Since the capacity of Pyk2 to augment ANF secretion and reduce cell spreading was accompanied by a significant increase in Pyk2 phosphorylation at Y402, we examined the effect of expression of two variant forms of Pyk2 on these processes (Fig. 4). Pyk2 Y402F contains a single amino acid substitution that abolishes Pyk2 autophosphorylation. Pyk2 Δ376 contains a deletion of the N-terminal 376 amino acids that results in high-level constitutive phosphorylation at Y402. Western blot analysis of NRVM lysates demonstrated that Pyk2 Y402F was well expressed but lacked detectable Y402 phosphorylation (Fig. 4a), whereas analysis of cell lysates from Pyk2-Δ376-expressing cells showed that Pyk2 Δ376 exhibited significant autophosphorylation (Fig. 4b). Unlike expression of wild-type Pyk2, expression of either Pyk2 Y402F or Pyk2 Δ376 in phenylephrine-stimulated NRVM did not augment phenylephrine-stimulated ANF secretion relative to phenylephrine-stimulated β-gal-infected control cells. Expression of either Pyk2 Y402F (Fig. 4d) or Pyk2 Δ376 (Fig. 4e) in phenylephrine-stimulated NRVM did not alter cell morphology relative to that of β-gal-infected control cells (Fig. 4c). The extent of cell spreading across the available surface area was comparable between the β-gal-expressing control cells (76.2%), the Pyk2-Y402F-expressing cells (82.3%), and Pyk2-Δ376-expressing cells (77.5%). These results indicated that autophosphorylation of Pyk2 was required but not sufficient to augment ANF secretion and to reduce cell spreading.

To determine whether the catalytic activity of Pyk2 is required for its effects on ANF secretion and cell morphology, NRVM were infected with Pyk2 K457A, which contains, in the activation loop, a substitution that abolishes kinase activity. Immunoblot analysis of the cell lysates showed that Pyk2 K457A was well expressed but with a significant decrease in detectable autophosphorylation, as assayed with the anti-pY402 specific antibody (Fig. 5a). Expression of the kinase-defective Pyk2 K457A in phenylephrine-stimulated NRVM inhibited ANF secretion in a dose-dependent manner relative to phenylephrine-stimulated NRVM control cells (Fig. 5a). Interestingly, loss of kinase activity did not restore spreading activity to stimulated myocytes. Myocytes expressing the kinase-defective Pyk2 K457A variant (Fig. 5c) exhibited reduced spreading relative to control cells (Fig. 5b; 50.5% of surface area vs. 63.8%, respectively). Notably, expression of the Pyk2 K457A variant in phenylephrine-stimulated myocytes produced significant morphological alterations, although different from those observed in myocytes expressing wild-type Pyk2. Phenylephrine-stimulated NRVM expressing wild-type Pyk2 were irregular with spindle-like morphology, whereas phenylephrine-stimulated myocytes expressing the Pyk2 K457A

variant were clustered and reduced in size with cells exhibiting a round to cuboidal morphology. These data indicated that Pyk2 catalytic activity was required for the augmentation of phenylephrine-stimulated ANF secretion but not necessary for the effect of Pyk2 on cell spreading.

Intracellular localization of Pyk2 Y402F, Pyk2 K457A, and Pyk2 Δ376

The effects of expression of the Pyk2 Y402F, Pyk2 K457A, or Pyk2 Δ376 variants relative to the wild-type Pyk2 might be mediated by alterations in subcellular localization. NRVM expressing Pyk2 Y402F, Pyk2 K457A, or Pyk2 Δ376 were fixed, stained, and analyzed with confocal microscopy. Similar to wild-type Pyk2, Pyk2 Y402F (Fig. 6a, b) and Pyk2 K457A (Fig. 6c, d) were localized in the cell periphery in non-stimulated myocytes, and phenylephrine stimulation resulted in their perinuclear accumulation. In contrast, Pyk2 Δ376 exhibited primarily perinuclear staining with a lesser degree of staining at the cell periphery in both non-stimulated and stimulated myocytes (Fig. 6e, f). Whereas ANF staining and myofibrillar organization was not altered by the expression of Pyk2 Y402F (Fig. 6b′, b″) or Pyk2 Δ376 (Fig. 6f′, f″) in phenylephrine-stimulated myocytes, the expression of Pyk2 K457A (Fig. 6d′, d″) eliminated ANF staining and also blocked phenylephrine-stimulated cell spreading. Cells were clustered with no structural or myofibrillar organization characteristic of phenylephrine-treated myocytes. Given that Pyk2 Δ376 exhibited predominantly perinuclear accumulation, whereas wild-type Pyk2, Pyk2 Y402F, and Pyk2 K457A exhibited perinuclear localization only following phenylephrine stimulation, the N-terminal region of Pyk2 can be suggested to play a role in this transition.

Effect of expression of an autonomous Pyk2 FERM domain

The inability of Pyk2 Δ376 expression to augment ANF secretion and its lack of morphological alterations pointed to a potential role for the N-terminal FERM domain of Pyk2 in these processes. Indeed, FERM domains mediate cellular signaling through protein-protein interactions at points of cytoskeletal linkages (Chishti et al. 1998). To investigate the role of the FERM domain of Pyk2 on phenylephrine-induced myocyte hypertrophy, NRVM were infected with increasing MOI of adenovirus encoding a HA-epitope-tagged FERM domain of Pyk2 (residues 39–367). Western blot analysis of NRVM cell lysates revealed that the autonomous Pyk2 FERM domain was well expressed and inhibited phenylephrine-stimulated ANF secretion in a dose-dependent manner relative to phenylephrine-stimulated control NRVM (Fig. 7a). Since an autoinhibitory function for the related N-terminal domain of FAK has been described (Dunty et al. 2004), we investigated the effect of the expression of the Pyk2 FERM domain on Pyk2 phosphorylation (Fig. 7b). NRVM were infected with the control β-gal or Pyk2 or were co-infected with Pyk2 and the Pyk2-FERM-expressing adeno-viruses. Immunoblots of cell lysates indicated that the expression of the Pyk2 FERM domain significantly inhibited the phosphorylation of Pyk2 on Y402 relative to that in NRVM only infected with Pyk2. The reduction in Pyk2 phosphorylation correlated directly with the reduction in the capacity of Pyk2 to increase ANF secretion.

Expression of the Pyk2 FERM domain might reduce Pyk2 phosphorylation by regulating its catalytic activity by direct association (Dunty and Schaller 2002). To investigate this possibility, NRVM were infected with equal MOIs of the control β-gal, Pyk2, or the Pyk2 FERM domain, or co-infected with Pyk2 and Pyk2 FERM. Cells were lysed and co-association-probed by immunoprecipitation. The Pyk2 FERM domain was immunoprecipitated by using anti-HA-specific antibody, and the presence of the full-length Pyk2 in the precipitate was analyzed by Western blot by using an anti-Pyk2 C-terminal-specific monoclonal antibody. Immunoblots indicated that full-length Pyk2 was co-immunoprecipitated with the autonomous Pyk2 FERM domain (Fig. 7c). Expression of the Pyk2 FERM domain (Fig. 7e) reduced cell spreading relative to phenylephrine-stimulated

control cells (Fig. 7d; 37.1% of surface area vs. 74.6%, respectively) and induced changes in cell morphology. Moreover, co-expression of the Pyk2 FERM domain together with Pyk2 in phenylephrine-stimulated myocytes (Fig. 7f) resulted in a greater reduction of cell spreading than that observed for phenylephrine-stimulated cells infected with the Pyk2 FERM domain alone (24.3% of surface area vs. 37.1%, respectively). Together, these results indicated that the FERM domain of Pyk2 associated with full-length Pyk2, inhibited Pyk2 autophosphorylation, blocked its capacity to increase ANF secretion, and probably mediated Pyk2 effects on cell morphology.

Discussion

The related cytoplasmic tyrosine kinases, Pyk2 and FAK, have been implicated in linking integrins and G-protein-coupled receptors to the regulation of cellular signaling pathways that lead to the induction and progression of cardiac hypertrophy (Bayer et al. 2001; Hirotani et al. 2004; Kodama et al. 2002, 2003; Taylor et al. 2000). In this report, we have examined the roles of Pyk2 and FAK in the adrenergic-mediated hypertrophic response. The major findings of this work are: (1) in phenylephrine-stimulated myocytes, expression of Pyk2 increases ANF secretion and reduces cell spreading, whereas expression of FAK attenuates ANF secretion but does not alter cell spreading; (2) Pyk2 and FAK exhibit a different subcellular distribution in response to phenylephrine stimulation; (3) autophosphorylation of Pyk2 is necessary but not sufficient for Pyk2 to augment ANF secretion and reduce cell spreading, whereas the catalytic activity of Pyk2 is required for the augmentation of ANF secretion but is not necessary for the ability of Pyk2 to reduce cell spreading; (4) the expression of the FERM domain of Pyk2 as an autonomous fragment inhibits phenylephrine-induced ANF production and reduces cell spreading; (5) the expression of the Pyk2 FERM domain reduces Pyk2 autophosphorylation and its ability to augment ANF secretion. These data suggest that Pyk2 and FAK function as important signaling effectors in cardiomyocytes and indicate that these kinases exert dissimilar effects on cellular signaling pathways involved in the temporal development of cardiac hypertrophy.

The increased expression of FAK in NRVM inhibits the phenylephrine-stimulated secretion of the hypertrophic marker ANF suggesting that FAK regulates some of the signaling mechanisms leading to the re-expression of fetal genes (Kovacic-Milivojevic et al. 2001; Taylor et al. 2000). Since ANF has been reported to exert an anti-hypertrophic effect (Hayashi et al. 2004), and since increased expression of FAK inhibits ANF secretion, FAK might mediate its pro-hypertrophic effects, in part, by inhibiting ANF secretion. In contrast, the increased expression of Pyk2 augments phenylephrine-stimulated ANF secretion and alters myocyte spreading suggesting that ANF secretion and cell spreading are regulated by distinct cellular signaling pathways. Indeed, the expression of Raf-1 can induce ANF-luciferase marker gene expression without affecting cell morphology (Thorburn et al. 1994, 1997). The differential subcellular distribution of Pyk2 and FAK in response to phenylephrine stimulation suggests that distinct effector and adaptor proteins are required to mediate their effects. Furthermore, these results suggest that Pyk2 and FAK have distinct and potentially opposing roles in the adrenergic-induced hypertrophic response. Indeed, Pyk2 and FAK have been proposed to function in an antagonistic manner in other cell types (Du et al. 2001; Zhao et al. 2000). In cardiomyocytes, studies have shown that Pyk2 plays an important role in the attachment of myocytes to the extracellular matrix, in cytoskeletal remodeling, and in apoptosis (Bayer et al. 2001; Chauhan et al. 1999; Rocic et al. 2001), and a relationship between cytoskeletal remodeling and apoptosis in cardiomyocytes has recently been established (Melendez et al. 2004). The results of these previous studies suggesting that Pyk2 is involved in cardiomyocyte remodeling is consistent with our findings of the decreased cell spreading and loss of myofibrillar structures in phenylephrine-stimulated Pyk2-expressing myocytes.

Different functional domains of Pyk2 appear to mediate its effects on phenylephrine-stimulated hypertrophic response. The Pyk2-stimulated augmentation of ANF secretion requires catalytic activity. Expression of a kinase-deficient Pyk2 functions with a dominant-negative effect inhibiting ANF secretion. The kinase-deficient Pyk2 K457Avariant exhibits a cellular distribution similar to that of wild-type Pyk2 and inhibits cell spreading as effectively as wild-type Pyk2 indicating that its effects on cell morphology are mediated by a domain other than the catalytic domain. Autophosphorylation alone is not sufficient to augment ANF secretion, as the expression of constitutively phosphorylated Pyk2 does not augment ANF secretion. Interestingly, the expression of Pyk2 Y402F does not produce morphological alterations in phenylephrine-stimulated myocytes. Similarly, the expression of an N-terminal deletion variant of Pyk2 does not promote the loss of myofibrillar structures observed in phenylephrine-stimulated myocytes expressing wild-type Pyk2. Although both variants, viz., Pyk2 Y402F and Pyk2 Δ376, ablate the effect of Pyk2 on cell spreading, the subcellular localization of these variants is not identical. Whereas Pyk2 Y402F exhibits a similar subcellular distribution to that of wild-type Pyk2, Pyk2 Δ376 is mainly localized in the perinuclear regions and to a less extent at the periphery of unstimulated myocytes. Phenylephrine stimulation results in near complete perinuclear localization. This suggests a potential novel regulatory role for the NH_2-terminal domain in the cellular localization of Pyk2.

The NH_2-terminal domain of Pyk2 contains a divergent FERM domain (Girault et al. 1998), a protein module linking cytoskeletal organization and signal transduction (Bretscher 1999). Expression of the FERM domain of Pyk2 as an autonomous fragment results in a dose-dependent inhibition of phenylephrine-stimulated ANF secretion and cell spreading. The ability of the Pyk2 FERM domain to inhibit ANF secretion is correlated with its ability to reduce Pyk2 phosphorylation. Immunoprecipitation experiments have revealed that the FERM domain co-immunoprecipitates with full-length Pyk2 suggesting that the FERM domain plays a direct role in regulating the activity of Pyk2. An earlier study was unable to demonstrate an interaction between the Pyk2 FERM domain and full-length Pyk2 (Dunty et al. 2004). Our results are consistent with those of a more recent study (Kohno et al. 2008) that demonstrates that an autonomously expressed Pyk2 FERM domain is capable of forming a Ca^{2+}/calmodulin-dependent heterodimer with full-length Pyk2 that, in turn, blocks Pyk2 homodimer formation and the resultant transphosphorylation of Pyk2 (Park et al. 2004). The ability of the autonomously expressed Pyk2 FERM domain to inhibit phenylephrine-stimulated cell spreading, together with the inability of Pyk2Δ376 to reduce cell spreading substantiates a central role for the FERM domain in Pyk2-mediated effects in myocyte hypertrophy.

In summary, the present study presents evidence that the two related cytoplasmic tyrosine kinases, Pyk2 and FAK, function as important signaling effectors in cardiomyocyte hypertrophy. The data indicate that expression of FAK and Pyk2 exerts different effects on the α_1-adrenergic-mediated alterations in cytoskeletal organization and expression of hypertrophic markers. Whereas FAK might be involved in promoting increases in cytoskeletal organization, Pyk2-mediated effects might be associated with stress-related signaling pathways leading to reduced cell spreading and decreased cytoskeletal organization. Moreover, the FERM domain of Pyk2 plays an important role in the capacity of Pyk2 to mediate its effects in phenylephrine-stimulated myocytes. An understanding of the specific functional significance of FAK and Pyk2 in the intracellular signaling mechanisms that mediate the pathology of cardiac hypertrophy could provide new insights leading to the development of effective therapeutic approaches for inhibiting the progression to heart failure.

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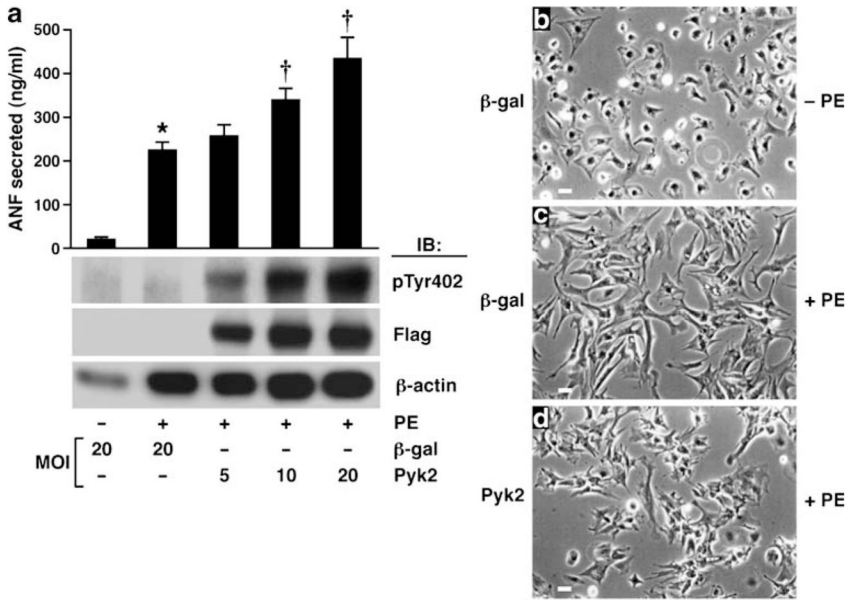

Fig. 1.

Effects of proline-rich tyrosine kinase 2 (*Pyk2*) expression on hypertrophic marker atrial natriuretic factor (*ANF*) secretion and cell spreading. **a** NRVM were infected with adenoviruses expressing Pyk2 or β-galactosidase (*β-gal*) at the indicated multiplicity of infection (*MOI*). Cells were cultured in serum-free media with (+) or without (−) 100 μM phenylephrine (*PE*) for 48 h. ANF was quantitated by enzyme-linked immunosorbent assay (ELISA). Data are depicted as means±SE, n=6 for each group; *P<0.05 comparing phenylephrine-stimulated control cells with non-stimulated control cells, $^{\dagger}P$<0.05 comparing phenylephrine-stimulated Pyk2-infected cells with phenylephrine-stimulated β-gal-infected cells. Cell lysates were immunoblotted with the indicated antibodies (*pTyr402* antibody specific for Pyk2 phosphorylated on Tyr402, *Flag* anti-FLAG M2 monoclonal antibody, *β-actin* anti-β-actin antibody). **b**–**d** Phase contrast images. **b** NRVM infected (MOI=20) with adenovirus encoding β-gal cultured in serum-free medium without phenylephrine (−*PE*). **c** NRVM infected with adenovirus encoding β-gal cultured in serum-free medium with 100 μM phenylephrine (+*PE*). **d** NRVM infected with adenovirus encoding Pyk2 cultured in serum-free medium with 100 μM phenylephrine (+*PE*). Observations were repeated in triplicate from more than four separate experiments. *Bars* 10 μm

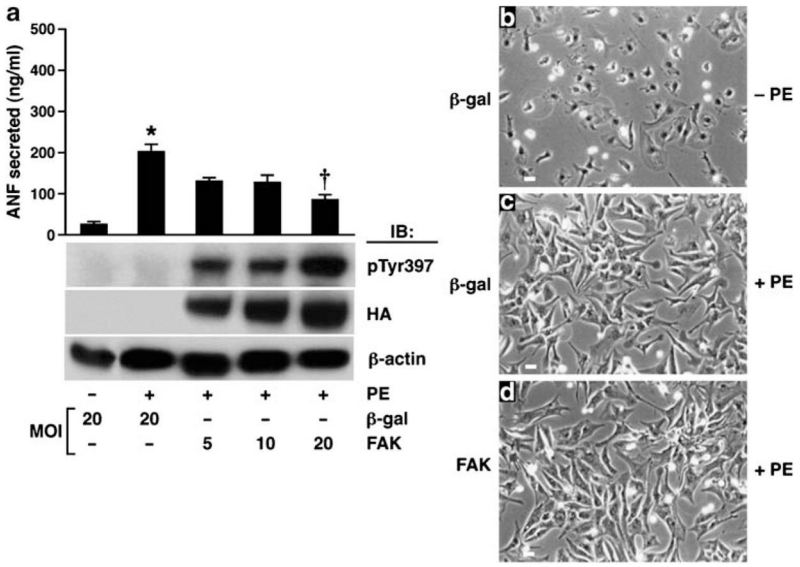

Fig. 2.

Effects of focal adhesion kinase (*FAK*) expression on ANF secretion and cell spreading. **a** NRVM were infected with adenovi-ruses expressing FAK or β-gal at the indicated MOI. Cells were cultured in serum-free media with (+) or without (−) 100 μM phenylephrine (*PE*) for 48 h. ANF was quantitated by ELISA. Data are depicted as means±SE, n=6 for each group. *P<0.05 comparing phenylephrine-stimulated control cells with non-stimulated control cells, $^{\dagger}P$<0.05 comparing phenylephrine-stimulated FAK-infected cells with phenylephrine-stimulated β-gal-infected cells. Cell lysates were immunoblotted with the indicated antibodies (*pTyr397* antibody specific for FAK phosphorylated on Tyr397, *HA* affinity-purified antibody to the HA epitope tag, *β-actin* anti-β-actin antibody). **b**–**d** Phase-contrast images. **b** NRVM infected (MOI=20) with adenovirus encoding β-gal cultured in serum-free medium without phenylephrine (−*PE*). **c** NRVM infected (MOI=20) with adenovirus encoding β-gal cultured in serum-free medium with 100 μM phenylephrine (+*PE*). **d** NRVM infected (MOI=20) with adenovirus encoding FAK cultured in serum-free medium with 100 μM phenylephrine (+*PE*).. Observations were repeated in triplicate from more than four separate experiments. *Bars* 10 μm

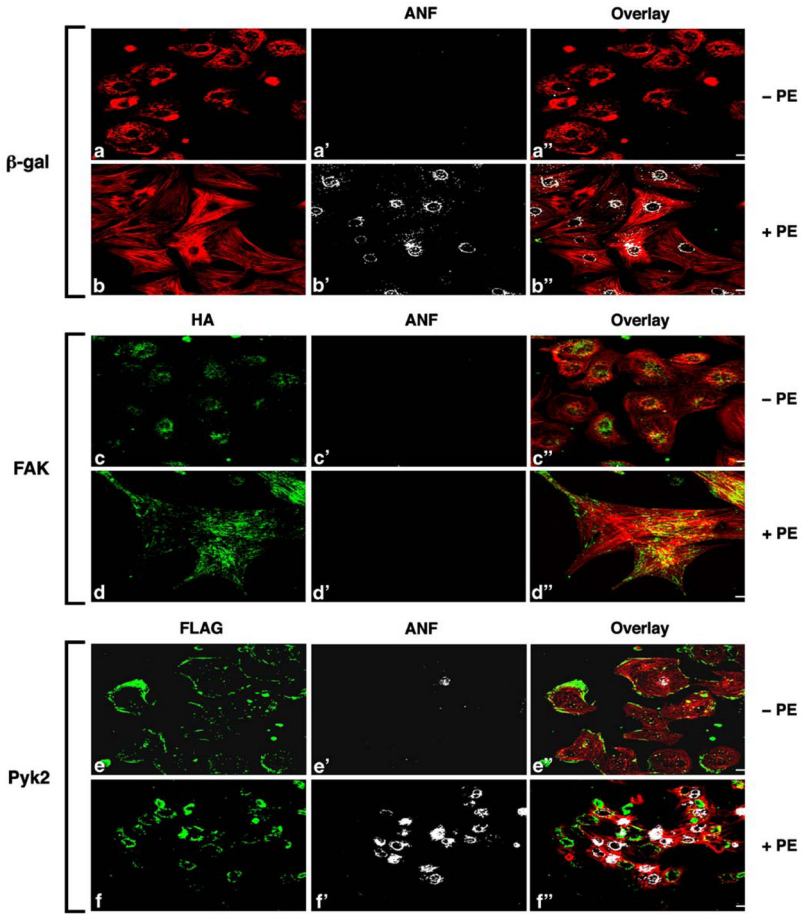

Fig. 3.

Differential localization of Pyk2 and FAK. NRVM were infected (MOI=20) with adenovirus encoding the control β-gal (**a**, **b**), HA-epitope-tagged (*HA*) FAK (**c**, **d**), or FLAG-epitope-tagged (*FLAG*) Pyk2 (**e**, **f**). Cells were fixed and stained with an anti-HA-FITC-conjugated goat anti-rabbit IgG (*green*) to detect expression of FAK (**c**, **d**). Pyk2 expression was detected with an anti-FLAG monoclonal antibody followed by FITC-conjugated goat anti-mouse IgG (*green* in **e**, **f**). ANF expression (*white*) was detected with an anti-ANF polyclonal antibody followed by a Cy-5 conjugated goat anti-rabbit IgG (**a′-f′**). Myofibrillar organization was assessed by staining with rhodamine-phalloidin (*red* in **a″-f″**). Images are representative of four independent experiments performed in duplicate *Bars* 10 μm

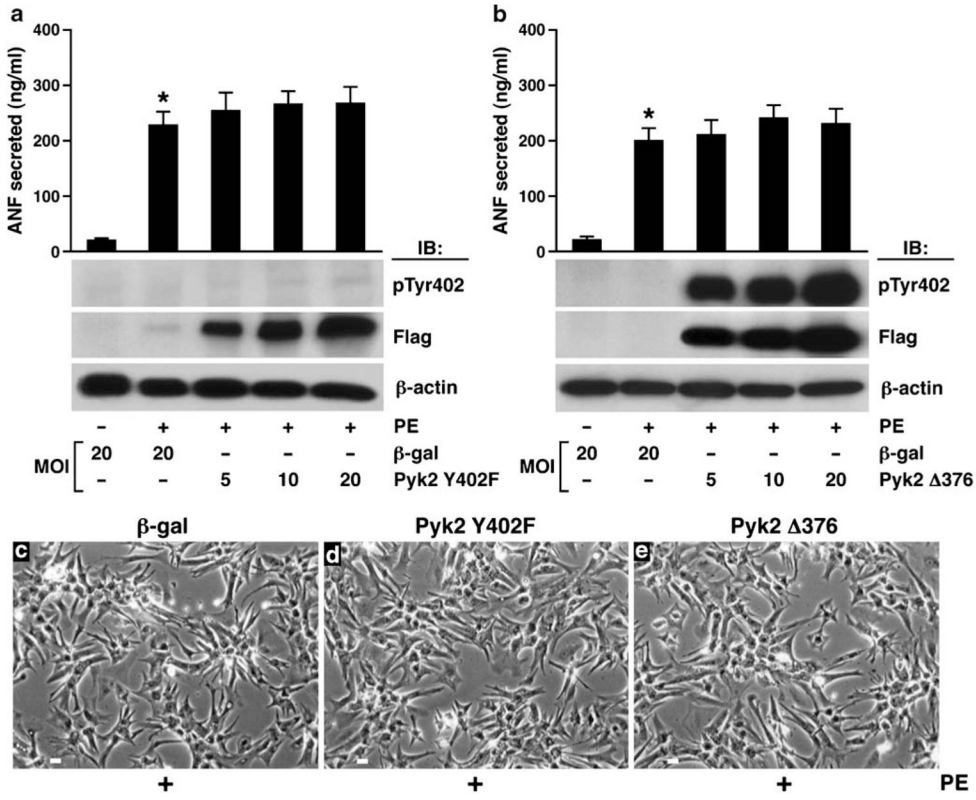

Fig. 4.

Autophosphorylation is required but not sufficient for Pyk2 to augment ANF secretion and to reduce cell spreading. **a** NRVM infected with adenovirus encoding FLAG-epitope-tagged Pyk2 Y402F or β-gal were cultured in serum-free media with (+) or without (−) phenylephrine (*PE*), and ANF was quantitated by ELISA. **b** NRVM infected with adenovirus encoding FLAG-epitope-tagged Pyk2 Δ376 or β-gal were cultured in serum-free media with or without phenylephrine, and ANF was quantitated by ELISA. Data are depicted as means ±SE, n=6 for each group, *P<0.05 comparing phenylephrine-stimulated β-gal-infected cells with non-stimulated β-gal-infected cells. Cell lysates were immunoblotted with the indicated antibodies. **c**–**e** Phase-contrast images of NRVM cultured in serum-free medium with 100 μM phenylephrine. **c** NRVM infected (MOI=20) with adenovirus encoding β-gal. **d** NRVM infected (MOI=20) with adenovirus encoding Pyk2 Y402F. **e** NRVM infected (MOI=20) with adenovirus encoding Pyk2 Δ376. Observations were repeated in triplicate from more than four separate experiments. *Bars* 10 μm

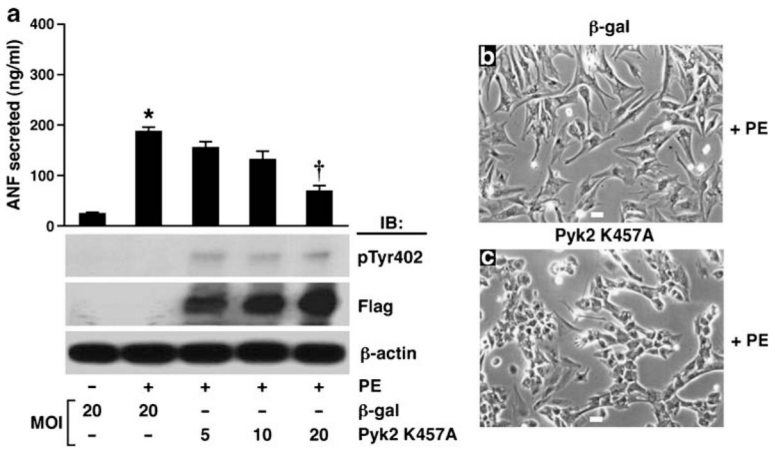

Fig. 5.

Expression of Pyk2 K457A inhibited ANF secretion and reduced cell spreading. **a** NRVM were infected at the indicated MOI with Pyk2 K457A or β-gal, cultured in serum-free media with (+) or without (−) phenylephrine (*PE*) for 48 h, and ANF production was quantitated by ELISA. Data are depicted as means±SE, n=6 for each group, *P<0.05 comparing phenylephrine-stimulated β-gal-infected cells relative to non-stimulated β-gal infected cells, $^{\dagger}P$<0.05 comparing phenylephrine-stimulated Pyk2-K457A-infected cells with phenylephrine-stimulated β-gal-infected cells. Cell lysates were immunoblotted with the indicated antibodies. **b**, **c** Phase-contrast images of NRVM cultured in serum-free medium with 100 μM phenylephrine. **b** NRVM infected (MOI=20) with β-gal. **c** NRVM infected (MOI=20) with Pyk2 K457A. Images are representative of four separate experiments. *Bars* 10 μm

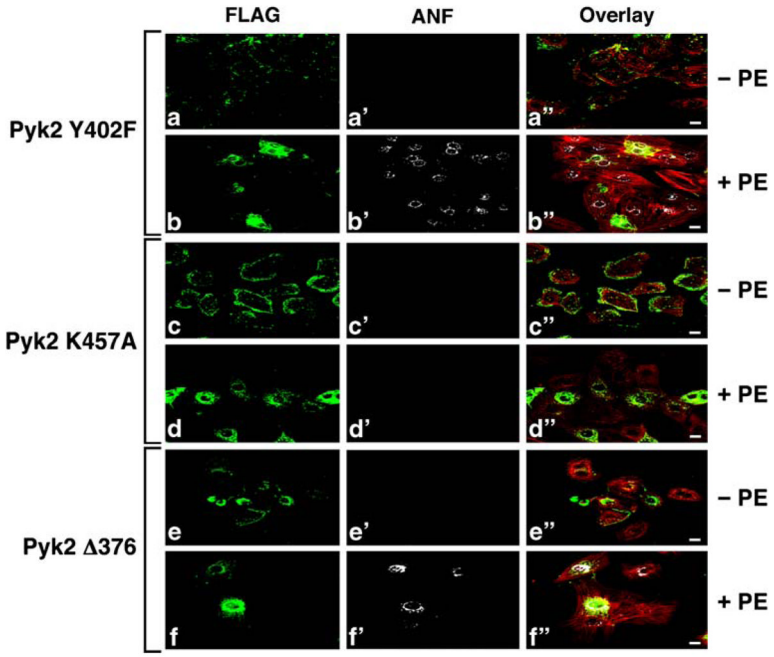

Fig. 6.

Localization of Pyk2 Y402F, Pyk2 K457A, and Pyk2 Δ376 in NRVM. NRVM were infected (MOI=20) with adenovirus encoding FLAG epitope-tagged Pyk2 Y402F (**a**, **b**), Pyk2 K457A (**c**, **d**), or Pyk2 Δ376 (**e**, **f**) and maintained in serum-free medium with (+) or without (−) phenylephrine (*PE*). Cells were fixed and stained with anti-FLAG antibody followed by FITC-conjugated goat anti-mouse IgG to detect the expression (*green*) of Pyk2 Y402F (**a**, **b**), Pyk2 K457A (**c**, **d**), or Pyk2 Δ376 (**e**, **f**). Cells were stained with anti-ANF antibody (**a′–f′**) followed by Cy-5-conjugated goat anti-rabbit IgG to detect ANF (*white*). Myofibrillar organization was assessed by staining with rhodamine-phalloidin (*red* in **a″–f″**). Images are representative of four independent experiments performed in duplicate. *Bars* 10 μm

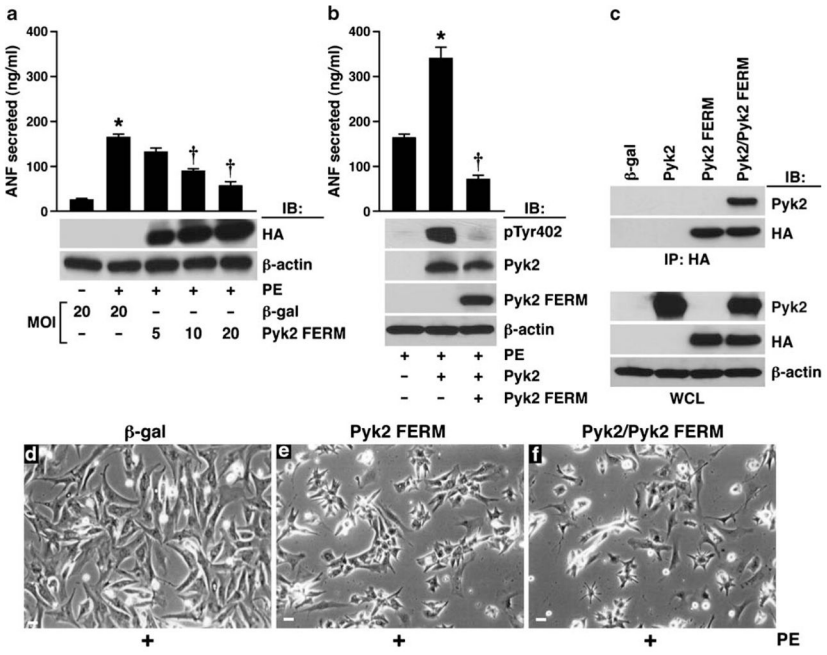

Fig. 7.

The Pyk2 FERM domain associates with Pyk2 and inhibits its activity. **a** NRVM were infected at the indicated MOI with adenovirus encoding the Pyk2 FERM domain or β-gal, cultured in serum-free media with (+) or without (−) phenylephrine (*PE*) for 48 h, and ANF production quantitated by ELISA. Data are depicted as means±SE, **P*<0.05 comparing phenylephrine-stimulated control cells with non-stimulated control cells, †*P*<0.05 comparing stimulated Pyk2-FERM-expressing cells with phenylephrine-stimulated β-gal-infected cells. **b** NRVM were uninfected, infected (MOI=20) with adenoviruses expressing Pyk2 (MOI=20) or co-infected with Pyk2 (MOI=20) and the Pyk2 FERM domain (MOI=20). ANF production was quantitated by ELISA. Data are depicted as means ± SE, *n*=6 for each group, **P*<0.05 comparing Pyk2-infected cells with control-stimulated cells, †*P*<0.05 comparing phenylephrine-stimulated co-infected cells to Pyk2-expressing cells. Cell lysates were immunoblotted with the indicated antibodies. **c** Myocytes were infected (MOI=20) with adenoviruses encoding β-gal, FLAG-tagged Pyk2, or HA-tagged Pyk2 FERM, or co-infected with Pyk2 (MOI=20) and the Pyk2 FERM domain (MOI=20) and immunoprecipitated by using anti-HA-specific antibody. Immunoprecipitates or whole cell lysates were immunoblotted with the indicated antibodies. **d–f** Phase-contrast images of NRVM cultured in serum-free medium with 100 μM phenylephrine. **d** NRVM infected with β-gal. **e** NRVM infected with the Pyk2 FERM domain. **f** NRVM co-infected with Pyk2 and the Pyk2 FERM domain. Images are representative of four separate experiments. *Bars* 10 μm