Intracellular Localization and Functional Effects of P21-Activated Kinase-1 (Pak1) in Cardiac Myocytes

Yunbo Ke, Lynn Wang, W. Glen Pyle, Pieter P. de Tombe, R. John Solaro

Abstract—We investigated intracellular localization and substrate specificity of P21-activated kinase-1 (Pak1) in rat cardiac myocytes. Pak1 is a serine/threonine protein kinase that is activated by Rac1/Cdc42 and important in signaling of stress responses. Yet the localization and in vivo function of Pak1 in heart cells is poorly understood. Studies reported here indicate that Pak1 physically interacts with protein phosphatase 2a and localizes to the Z-disk, cell membrane, intercalated disc, and nuclear membrane of adult rat heart myocytes. We compared levels of phosphorylation of cardiac troponin I (cTnI) in control myocytes with phosphorylation of cTnI and myosin binding protein C (C-protein) in myocytes with increased Pak1 phosphorylation. The increase in activity was induced by infection of myocytes with a recombinant adenovirus (AdPak1) containing cDNA for a constitutively active Pak1. Control cells were infected with a virus (AdLacZ) containing LacZ. Basal levels of phosphorylation of cTnI and C-protein were relatively high in the myocytes infected with AdLacZ. However, phosphorylation of cTnI and C-protein in cells expressing constitutively active Pak1 was significantly reduced compared with those expressing LacZ. Measurement of Ca2+ tension relations in single myocytes demonstrated that this reduction in phosphorylation of cTnI and C-protein was associated with the predicted increase in sensitivity to Ca2+. Our data provide evidence for a novel pathway of phosphatase regulation in cardiac myocytes. (Circ Res. 2004;94:999-1006.)

Key Words: heart failure • phosphatase • troponin
Experiments reported here were aimed at testing whether expression of active Pak1 in situ in cardiac myocytes alters phosphorylation and regulatory function of cTnI. Our results demonstrate relatively high levels of endogenous Pak1 in heart cells. By using adenoviral transfer of Pak1 into adult cardiac myocytes, we demonstrate that expression of active Pak1 reduces phosphorylation of cTnI and increases myofilament sensitivity to Ca^{2+}. Our data indicate that rather than causing phosphorylation of cTnI, Pak1 promotes dephosphorylation of cTnI, most likely through the activation of PP2A.

**Materials and Methods**

**Isolation and Culture of Ca^{2+}-Tolerant Cardiac Ventricular Myocytes**

Cardiac ventricular myocytes were isolated from the hearts of adult male rats, 2 to 3 months of age, using a collagenase perfusion method, as previously described. All experiments were performed according to institutional guidelines concerning the care and use of experimental animals. In cell culture, we used freshly isolated adult rat cardiac myocytes to settle onto cover slips (Fisherbrand No. 1D 12-545-83) in a 12-well tissue culture plate. The cover slips were precoated with mouse laminin at 10 μg/mL for 2 hours. The cells were first cultured for 2 hours in DMEM (Sigma D6421) plus 10% FBS. After we changed the medium to serum-free DMEM, the cells were cultured overnight.

**AdPak1 Construction, Viral Amplification, and Plaque Assays**

To prepare recombinant adenovirus that expresses constitutively active Pak1 (AdPak1), we used the polymerase chain reaction method to tag the human Pak1 cDNA with an HA epitope (YPYD-VPDY) at the N-terminal region and next to the translational initiation codon. Threonine 423 was mutated to glutamic acid in Pak1 cDNA to convert the Pak1 protein into a constitutively active form. The cDNA was cloned into a shuttle vector pAdCMV to obtain pAdCMVPak1. The AdPak1 was made by homologous recombination between pAdCMVPak1 and the viral backbone DNA DL7001. The lysate of virus amplified from a clone was used to infect the cells cultured in 100-mm dishes, and 5 to 10 large dishes were harvested 28 to 35 hours after infection. The lysates from cells cultured in 100-mm dishes, and 5 to 10 large dishes stored at −70°C were used for immunoprecipitation experiments. To evaluate viral titers, we infected 100-mm dishes with AdPak1 or AdLacZ for 8 hours, in which the level of transgene expression was substantial, DMEM was replaced with Na-HEPES phosphate-free buffer (in mmol/L, CaCl_2 1.0, KCl 4.8, MgSO_4 1.2, NaCl 132, HEPES 10, Na pyruvate 2.5, and glucose 10; pH 7.4) with 0.5 mCi [32P] orthophosphate for 30 minutes at room temperature. The cells were then washed twice with the Na-HEPES solution with 1 mmol/L CaCl_2. Two minutes after adding isoproterenol or vehicle, we added an equal volume of SDS-stop solution (in mmol/L, DTT 1, Tris-HCl 150, EDTA 3, with 6% SDS, 15% glycerol, and a trace of bromophenol blue). Before analysis by SDS-PAGE, the samples were boiled for 10 minutes. Gel electrophoresis was performed using either a 12% or a linear 5% to 20% polyacrylamide gradient gel, as previously described. An aliquot of cells containing 50 μg protein, as determined using the Lowry method, was analyzed by SDS-PAGE, as previously described.

**Labeling of Proteins for Immunofluorescence and Western Blotting**

For immunolabeling, cover slips containing adult rat cardiac myocytes were washed twice with PBS, and the cells were fixed with 2% paraformaldehyde. The cells were washed twice (5 minutes) and then incubated with 2% paraformaldehyde. The cells were washed twice (5 minutes) with PBS containing 0.25% NH_4Cl, 0.01% saponin, and 0.02% NaN_3. The cells were then washed with PBS containing 0.5% BSA, 0.01% saponin, and 0.02% NaN_3. The primary antibody was added in coating reagent and incubated for 30 minutes. The cells were washed three times in PBS containing 0.05% saponin and 0.02% NaN_3, incubated in secondary antibody for 15 minutes in PBS containing 0.05% BSA, 0.01% saponin, and 0.02% NaN_3, and then washed three times (for 5 minutes) with PBS containing 0.01% saponin and 0.02% NaN_3. Anti-Pak1 (α-Pak) polyclonal antibody of rabbit origin was from Santa Cruz Biotechnology (No. sc-6110). For immunofluorescence studies, we used a 1:50 dilution, and for Western blot analysis, a 1:200 dilution. The secondary antibody (1:200 dilution) was FITC-conjugated anti-rabbit IgG of goat origin (Sigma, No. F-9887). Rhodamine-conjugated phalloidin was purchased from Sigma (No. p 1951).

Images were acquired using a BioRad laser-scanning confocal microscope Radiation 2000 equipped with a ×60 water immersion objective. A 488-nm and a 568-nm beam from an argon-krypton laser were used for excitation. Detection of the emissions from green and red fluorescence was through HQ515/30 and HQ590/70 filters, respectively. The collected images were processed using LaserPix version 4.0 (BioRad).

**Tension-Ca^{2+} Relations in Skinned Cardiac Myocytes**

Adult rat cardiac myocytes were infected with AdPAK1 or AdLacZ at moi of 100. After 15 hours in culture, isolated myocytes were buffer and lyophilized. Kinase activity of expressed Pak1 was determined in 50-μL reaction mixtures, which contained 0, 0.1, or 1 μg of the purified Pak1 protein, 10 μCi of (γ-32P)ATP, and 5 μg of myelin basic protein in kinase assay buffer (50 mmol/L HEPES [pH 7.3], 10 mmol/L MgCl_2, 2 mmol/L MnCl_2, 1 mmol/L dithiothreitol, 0.05% Triton X-100). The mixture was incubated at 30°C for 30 minutes. Samples were resolved by SDS-PAGE on 12% gels and processed for autoradiography. We also immunoprecipitated expression HA-Pak1 from infected cardiac myocytes and endogenous Pak1 from myocardium. The precipitated proteins was resolved by SDS-PAGE and probed with PP2A antibody (Santa Cruz, No. sc-6110).
washed for 15 minutes with ice-cold high-relax (HR) buffer at pH 7.0 containing, in mmol/L, EGTA 10, CaCl$_2$ 0.025, MOPS 20, KCl 50, MgCl$_2$ 6.8, phosphocreatine 12, and Na$_2$ ATP 5. HR also contained 5 mg/mL leupeptin, 12.5 mg/mL pepstatin, and 0.25 mmol/L PMSF, pH 7.0. At this stage, 1% Triton X-100 was added to HR to remove membranes. The mixture containing detergent-extracted cells was centrifuged at 10 000 g for 10 minutes, and the pellet was washed in ice-cold HR. The suspension was centrifuged again, and the resulting pellet was resuspended in ice-cold HR buffer. The detergent-extracted cells were kept on ice up to a maximum of 8 hours until single myocytes were attached with silicone glue to micropipettes, as previously described.21 One micropipette was mounted to a force transducer (Cambridge model 403A), and the other to a high-speed motor (Cambridge model 308). Sarcomere length was set to 2.20 μm and monitored using custom-designed software (Labview, National Instruments); tension was computed from the cross-sectional area, as previously described. 21 Ca$^{2+}$ sensitivity was expressed as EC$_{50}$ (Ca$^{2+}$ concentration at half-maximal activation). The first and last contractions were at maximally activating Ca$^{2+}$ to assess any functional decline. Only cells retaining >80% of their initial maximum contraction were kept for analysis.

Data Analysis
Data are presented as mean±SEM. The significance of differences between means was evaluated with 2-way ANOVA (for repeated measures, when appropriate). Values of $P<0.05$ were considered statistically significant.

Results
Expression of Pak1 in Adult Rat Cardiac Myocytes Is Abundant and Localized to Z-Discs
To investigate subcellular localization of endogenous Pak1 in adult rat cardiac myocytes, we used immunofluorescence labeling and confocal microscopy. The saponin-permeabilized cardiac myocytes were probed with an antibody for Pak1. In control cells, rabbit and calf serum was used instead of Pak1 antibody. As displayed in Figure 1, left, Pak1 staining produced a striated pattern and demonstrated localization to cell and nuclear membranes. The result shown in Figure 1 is typical of >100 cells examined in this way. We observed no striations in control myocytes incubated with rabbit or calf serum without Pak1 antibody (data not shown). Western blot analysis for Pak1 detected a single band of 68 kDa in homogenates prepared from rat and mouse ventricle. A sarcomeric pattern was also observed when myocytes were stained with rhodamine-conjugated phalloidin in addition to Pak1 (Figure 1, right). The Pak1 signals are green, and actin signals demonstrated by the rhodamine-conjugated phalloidin staining are red. Yellow staining revealed a striated pattern of actin-Pak1 colocalization and indicates a sarcomeric localization of the Pak1. This localization is additionally demonstrated by evidence displayed in Figure 2, which shows data similar to those in Figure 1, but at higher magnification. In Figure 2, the Pak1 signals (green on left) became yellow when superimposed with signals produced by rhodamine-conjugated phalloidin (red on right). Yellow staining forms stripes on each side of a relatively dark transverse band formed by the actin-free H-zone on the sarcomere. These results indicate that Pak1 localizes to the Z-disc. In Figure 3, we show immunofluorescence labeling of HAPak1 protein in a myocyte infected with AdPak1. In this case, sarcomeric localization of AdPak1 is not evident, indicating that the active Pak1 is situated in a cytosolic localization.

Increased Pak1 Activity in Myocytes Is Associated With Dephosphorylation of Troponin I and Increased Myofilament Ca$^{2+}$ Sensitivity
We tested whether the adenoviral transfer of HAPak1 DNA into cardiac myocytes resulted in expression of a constitutively active Pak1 with functionally significant effects. Figure
Figure 2. Localization of Pak1 signals as striations in the middle of phalloidin bands. A, Adult rat cardiac myocytes were incubated with anti-Pak1 antibody at 1:50. B, Anti-pak1 antibody at 1:50 plus rhodamine-conjugated phalloidin at 1:1000. The experimental conditions were the same as described in Figure 1, except magnification is ×2.

Figure 3. Expression of constitutively active HA-AdPak1 in adult cardiac myocytes. Myocytes in culture were infected with AdPak1 overnight, as described in Materials and Methods. The HAPak1 was detected by FITC-conjugated antibody (Roche Applied Science; No. 1 988 506). The nuclei were stained blue by DAPI (4',6'-diamidino-2-phenyindole).

Figure 4 shows data demonstrating the expression level and activity of recombinant Pak1. Western blot analysis showed expression of HAPak1 protein in the cardiac myocytes (Figure 4A). The activity of the HAPak1 is demonstrated by data depicted in Figure 4B, which illustrate that the recombinant Pak1 protein isolated from infected myocytes was able to phosphorylate an exogenous substrate (myelin basic protein) in vitro. Pak1 was also able to phosphorylate cTnI in vitro (Data not shown). Data in Figure 4B agree with earlier reports on the ability of Pak1 to phosphorylate MBL and to autophosphorylate. To test whether the constitutively active Pak1 modifies the state of phosphorylation of myofilament proteins in situ, we compared 32P-orthophosphate incorporation into myofilament proteins in cardiac myocytes infected with AdLacZ or AdPak1. Lane 1 of Figure 5, which served as a standard, displays isoproterenol-induced incorporation of 32P into cTnI and myosin binding protein C in control myocytes that were not treated with the adenoviral constructs. Lane 2
shows the level of phosphorylation of cTnI and myosin binding protein C in cells infected with AdLacZ and in a basal state. However, as demonstrated in lane 3 of Figure 5, infection with AdPak1 significantly reduced both cTnI and myosin binding protein C phosphorylation. Incorporation of 32P into myosin light chain 2 and cTnT was essentially the same in all the cells.

To determine whether the dephosphorylation of cTnI translated into a functional effect, we measured the isometric tension as a function of Ca2+ in single myocytes that had been infected with the recombinant adenovirus expressing either Pak1 or LacZ before detergent extraction. Adequate evidence indicates that phosphorylation of cTnI reduces the Ca2+ sensitivity of the myofilaments.1-3 Data summarized in Figure 6 show that Ca2+ sensitivity of tension was significantly higher in myofilaments from AdPak1-infected myocytes (EC50, 0.87±0.14 μmol/L) compared with AdLacZ controls (EC50, 1.43±0.08 μmol/L). Maximum developed isometric tension tended to be greater in myofilaments from AdPak1-infected myocytes (32.2±9.8 mN) compared with AdLacZ-infected myocytes (23.1±5.4 mN), but this trend did not reach statistical significance. The Hill coefficient (3.8±0.6 for AdLacZ and 3.9±0.8 for AdPak1-infected myocytes) was not different between treatment groups. Myofilaments of uninfected control myocytes (n=19) not subjected to culture demonstrated Ca2+ activation parameters (EC50, 1.40±0.03 μmol/L; maximum tension, 24.5±1.9 mN/mm2; and Hill n, 4.1±0.3) that were not significantly different from those of myocytes infected with AdLacZ.

Results reported in Figure 6 demonstrate that the reduction in cTnI phosphorylation induced by Pak1 is associated with the expected functional effect of enhanced sensitivity to Ca2+.

Figure 4. Expression and activity of constitutively active HAPak1 in adult rat cardiac myocytes. Myocytes were infected with either AdLacZ or HA-AdPak1 in Tyrode’s solution, as described in Materials and Methods. A, Detection of the expressed Pak1 by antibody against HA tag. Lane 1, Myocytes infected with AdLacZ. Lane 2, Myocytes infected with AdPak1. B, Activity of Pak1 purified from infected myocytes demonstrated by phosphorylation of myelin basic protein. Lanes 1, 2, and 3 contain 0, 0.2, and 2 μg of Pak1 protein, respectively. The upper band results from Pak1 autophosphorylation, and the lower band from Pak1-induced phosphorylation of myelin basic protein. See Materials and Methods for details.

Figure 5. Active Pak1 inhibits phosphorylation of cTnI in adult rat cardiac myocytes. Myocytes were first infected with either AdPak1 or AdLacZ at moi of 100 and incubated in 32P to label the nucleotide pool. Myocyte proteins were subsequently separated by SDS-PAGE (12%) and subjected to autoradiography, as described in Materials and Methods. Lane 1, Positive control demonstrating phosphorylation of myosin binding protein C (MyB Prot C) and cTnI when myocytes without viral infection were treated with 1 μmol/L isoproterenol. Lane 2, Basal phosphorylation of cTnI, cTnT, ventricular myosin light chain 2 (MLC2v), and MyB Prot C in myocytes infected with AdLacZ. Lane 3, Demonstration of reduction in phosphorylation of cTnI and MyB Prot C in myocytes infected with AdPak1. The same results were obtained in 5 separate experiments.
Inasmuch as PP2A is the major phosphatase dephosphorylating PKA sites on cTnI, we tested whether HAPak1 interacts with PP2A. We infected 911 cells in culture with AdPak1, purified the expressed protein by affinity chromatography, and probed for the copurification of PP2A with an antibody to the 36-kDa catalytic subunit of PP2A. As illustrated in Figure 7A, PP2A was copurified with HAPak1. As additional evidence that HAPak1 is able to activate PP2A, we probed an extract of cardiac myocytes infected with either AdLacZ or AdPak1 with an antibody that detects the phosphorylation of Y307 of PP2A catalytic subunit. Dephosphorylation of Y307 is associated with activation of PP2A. The results are depicted in Figure 7B. In myocytes infected with AdLacZ, we were able to detect a reaction of the antibody. However, in myocytes infected with AdPak1, we could not detect phosphorylation of Y307, which indicates activation of the catalytic subunit of PP2A. We also infected cardiac myocytes with AdPak1 and immunoprecipitated AdPak1 with the HA antibody. The immunoprecipitated proteins were then probed with an antibody to PP2A. As illustrated in Figure 7C, lane 1, PP2A copurified with HAPak1. Endogenous myocardial Pak1 also coimmunoprecipitated with PP2A, as illustrated by the data in Figure 7 (lanes 2 and 3). In this case, immunoprecipitation was carried out using an antibody to Pak1, and the resulting proteins were probed by Western blot analysis with antibodies to Pak1 and PP2A.

**Discussion**

Data presented here are the first to demonstrate a significant functional effect of in situ Pak1 activation in cardiac myocytes. Our data are also novel in demonstrating dephosphorylation of cTnI and myosin binding protein C and enhanced myofilament Ca\(^{2+}\)/H\(_{11001}\) sensitivity associated with increased Pak1 activity. These effects of Pak1 activation may be attributable to increased activity of phosphatase, most likely PP2A. Our results also alter thinking about the role of Pak1 in regulation of cardiac function in that they indicate that previously reported \(\text{Ca}^{2+}\)-sensitizing effects of direct in vitro phosphorylation of cTnI by Pak3 on myofilament tension occur by a different mechanism in the case of in situ activation of Pak1.
However, Pak3 has been identified to be expressed in rat and pig hearts. Pak2, a ubiquitously expressed member of the Pak family of proteins, may also be expressed in heart. The catalytic domains of Pak1, Pak2, and Pak3 share >93% homology, but N-terminal regions, believed to be of importance in cellular localization, may possess structural differences. Thus, we cannot rule out the possibility that functional effects of Pak isoforms involve a balance of direct phosphorylation and indirect activation of phosphatase activity. Moreover, it is also important to point out that both Rac1 and cdc42 have immediate downstream effectors other than Paks in cardiac myocytes.

An important question is whether Pak1 is involved in signaling pathways in which dephosphorylation of cTnI is known to occur. It has been known for some time that cholinergic receptor agonists and adenosine receptor agonists induce an antiadrenergic effect and an associated dephosphorylation of substrates, including cTnI. Early studies indicated that the mechanism for this effect of adenosine or cholinergic agonists was a depression in adenyl cyclase activity, resulting in depressed cAMP levels and depressed activation of PKA. However, subsequent experiments concluded that the effect was attributable to stimulation of protein phosphatases with little or no change in cAMP concentrations. Moreover, in the case of adenosine, antiadrenergic effects on intracellular Ca2+ and contraction of cardiac myocytes could be blocked by phosphatase inhibitors. Along these lines, Liu and Hofmann demonstrated that antiadrenergic effects mediated by adenosine A1 receptors in the perfused heart were associated with carboxymethylation of the PP2A catalytic subunit, translocation of the holoenzyme, and dephosphorylation of both cTnI and phospholamban. Although these data strongly indicate that the antiadrenergic mechanism involves direct dephosphorylation of cTnI at the PKA sites by adenosine, it is also important to point out that both Rac1 and cdc42 have immediate downstream effectors other than Paks in cardiac myocytes.

The signaling pathway by which PP2A becomes activated remains unclear, although recent data indicate that PP2A activation may be mediated through G-guanyl cyclase p38 mitogen-activated protein kinase (MAPK) pathway. In cardiac myocytes in which Gβγ was blocked by pertussis toxin, dephosphorylation of cTnI at the PKA sites by adenosine did not occur. Similarly, inhibition of Gβγ by pertussis toxin prevented lysophosphatidic acid–induced cell spreading of fibroblasts. Zhang et al reported that coexpression of constitutively active forms of Rac and Cdc42, upstream regulators of Pak1 activity, in COS or HEK cells leads to the activation of p38. Moreover, Zhang et al demonstrated that Pak1 stimulated p38 activity and that a dominant-negative Pak1 depressed induction of p38 activation by both interleukin-1 and Rac/Cdc42. The conclusion drawn from these studies was that the initiation of the signaling cascade by Rac and Cdc42 is at the level of Pak1, which leads to the activation of p38 MAPK. Based on our present work and previously reported studies, we hypothesize that the signaling cascade activated in our experimental models consists of Gβγ stimulation of Rac or Cdc42, which in turn activate Pak1. Pak1 increases p38 MAPK activity, which stimulates PP2A-mediated dephosphorylation of cTnI. The importance of Pak1 in the regulation of phosphatase activity is evidenced by its highly conserved and ubiquitous nature. For example, PP2A in yeast is regulated by a phosphotyrosyl phosphatase activator, which is positioned downstream of Cla4P, the yeast homologue of Pak1.

In addition to adenosine, there are other potential pathways for physiological and pathological signaling that promote the activity and expression levels of Pak1. These include bradykinin, lysophosphatidic acid, activators of the Rac1 pathway, and stressors such as osmotic shock. In agreement with our findings of a relatively high level of expression of Pak1 in adult rat cardiac myocytes, Clerk and Sugden first reported high basal levels of Pak1 in neonatal cardiac myocytes. Clerk and Sugden also demonstrated an increased activity of Pak1 when the cells were stressed by hyperosmotic shock. Hearts of mice expressing constitutively active Rac1 also demonstrated an activation of Pak1 compared with nontransgenic controls.

Activation of phosphatase PP2A by Pak1 has potential implications in heart failure. Pak1 is downstream of Rac1, and there is evidence that the constitutive activation of Rac1 in a transgenic mouse model leads to dilated cardiomyopathy. Sussman et al suggested that this phenotype was mediated through a loss of Pak1 regulation by the focal adhesions. Pak1 is known to interact with focal adhesion complexes at the cell membrane, where it is targeted to paxillin. With activation of Rac1 signaling, Sussman et al report a movement of Pak1 from a cytosolic to a particulate location. The deregulation of focal adhesions was proposed to result in loss of cell adhesion and normal systolic function that leads to a dilated cardiomyopathy. Our data indicate that consideration must also be given to the possibility that the activity of regulatory proteins such as cTnI are also affected by altered Pak1 activation.

Acknowledgments
This work was supported by NIH Grants P01 HL 62426, R37 HL 22231, and R01 HL 64035. Y.K. was supported by T32 HL 07692 and W.G.P. by an American Heart Association Post-Doctoral Fellowship.

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Circ Res. published online December 11, 2003;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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