p21-Activated kinase-1 and its role in integrated regulation of cardiac contractility

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Sheehan KA, Ke Y, Solaro RJ. p21-Activated kinase-1 and its role in integrated regulation of cardiac contractility. Am J Physiol Regul Integr Comp Physiol 293: R963–R973, 2007. First published July 3, 2007; doi:10.1152/ajpregu.00253.2007.—We review here a novel concept in the regulation of cardiac contractility involving variations in the activity of the multifunctional enzyme, p21-activated kinase 1 (Pak1), a member of a family of proteins in the small G protein-signaling pathway that is activated by Cdc42 and Rac1. There is a large body of evidence from studies in noncardiac tissue that Pak1 activity is key in regulation of a number of cellular functions, including cytoskeletal dynamics, cell motility, growth, and proliferation. Although of significant potential impact, the role of Pak1 in regulation of the heart has been investigated in only a few laboratories. In this review, we discuss the structure of Pak1 and its sites of posttranslational modification and molecular interactions. We assemble an overview of the current data on Pak1 signaling in noncardiac tissues relative to similar signaling pathways in the heart, and we identify potential roles of Pak1 in cardiac regulation. Finally, we discuss the current state of Pak1 research in the heart in regard to regulation of contractility through functional myofilament and Ca\(^{2+}\)-flux modification. An important aspect of this regulation is the modulation of kinase and phosphatase activity. We have focused on Pak1 regulation of protein phosphatase 2A (PP2A), which is abundant in cardiac muscle, thereby mediating dephosphorylation of sarcomeric proteins and sensitizing the myofilaments to Ca\(^{2+}\). We present a model for Pak1 signaling that provides a mechanism for specifically affecting cardiac cellular processes in which regulation of protein phosphorylation states by PP2A dephosphorylation predominates.

Protein phosphatase 2A; sarcomeric proteins; Ca\(^{2+}\) flux proteins

CARDIAC CONTRACTILITY IS REGULATED through a number of signaling pathways that integrate to affect whole heart function and adaptation to intrinsic and extrinsic stressors. The dynamic balance of kinase and phosphatase activities represents a major mechanism for the control of the functional state of a number of cardiac regulatory proteins and thus the state of contractility from beat to beat, in adjustment to neurohumoral changes over time, and in disease states. Regulation of kinases such as cAMP-dependent protein kinase (PKA) and lipid-dependent protein kinase C (PKC) are relatively well known and include activation by receptor-mediated agonist stimulation, localization with target proteins, and inhibition of activity through various negative-feedback processes. On the other hand, although phosphatases such as protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) are established agents of antiadrenergic cellular response, the specific mechanisms regulating their activity are not completely elucidated. Finally, cellular morphology and structural proteins can mediate not only the size and shape of a cell but can contribute to functional signaling.

In this review, we focus on emerging evidence for p21-activated kinase 1 (Pak1) as a critical element in the regulation of cardiac contractility. We will consider the structure and activation mechanisms of Pak1, evidence for its function in noncardiac tissues, and how these signaling mechanisms and functional consequences are relevant in the heart. Finally, we will examine recent evidence for Pak1 as part of a novel signaling pathway in PP2A regulation and modulation of cardiac regulatory protein dephosphorylation in the context of integrated control of cellular function and morphology.

THE PAK FAMILY OF ENZYMES, PAK1 PROTEIN STRUCTURE, AND SITES OF ACTIVATION

Pak structure and molecular basis of activity. The Paks are a family of serine-threonine protein kinases exhibiting direct activation by the small GTPases Cdc42 and Rac1 (82, 91). The family of enzymes consists of two groups. Group I, including Pak1, Pak2, and Pak3, are the best characterized and have the greatest degree of sequence homology. The Group II Paks are not as well characterized, have significantly less homology overall, and lack Cdc42 and Rac1 binding domains (see Refs. 15 and 52 for reviews). The protein structure of Group I Paks in mammalian cells is highly conserved and shares substantial...
homology with yeast Cla4 and Ste20 (83). Pak1 (α-Pak) was first discovered in rat brain as a small GTPase-binding protein (82). Of both the Pak groups, Pak1 is reported to be the predominant isoform in muscle, including the heart (52). Pak1 is a 545-amino acid protein (Fig. 1) with a large NH2-terminal binding domain and inhibitory region (aa 70-149) and a COOH-terminal kinase region (aa 255-529) (77). Within the NH2-terminal region is a p21-binding domain (PBD; aa 70-113) and a COOH-terminal kinase region (aa 255-529) (77). Within the NH2-terminal region is a p21-binding domain (PBD; aa 70-113) with an embedded minimal sequence for Cdc42 and Rac-interactive binding (CRIB; aa 75-90) (65, 67, 103). Partially overlapping this segment is the autoinhibitory region (aa 83-149) that forms part of the “inhibitory switch” and participates in inhibition of COOH-terminal kinase activity in the inactive dimerized conformation (77, 91). On the COOH-terminal side of the inhibitory region is an acidic region whose functional significance is not known.

The overall peptide sequence contains five proline-rich regions (82) of the (PXXP) SH3-binding motif that are significant in mediating proliferative responses. The first has been identified as a binding site for the SH3-containing Nck adapter protein (aa 12-18) (17, 39). COS-7 cell stimulation with platelet-derived growth factor induces Nck binding to Pak1, functionally linking Pak1 and receptor tyrosine kinases in proliferation and cell growth. In vitro, phosphorylation of the Akt site, Ser-21, attenuates the interaction between Nck and Pak1. The second SH3 domain (aa 40-47) interacts with the Grb2 adapter protein and mediates the interaction of Pak1 with the epidermal growth factor receptor in vitro and in vivo (95). In other studies, Ser-21 phosphorylation promotes Nck interaction and cellular migration in nonmuscle cells (139, 140). Finally, phosphorylation of the Cdc2/Cdk5 site (Thr-212) is involved in neuron microtubule dynamics (10, 138). Additional autophosphorylation sites have been reported at Ser-57, Ser-144, Ser-149, and Ser-198 (25), but their functional significance is not well known. Amino acids 182-203 constitute the fourth proline-rich region that represents a noncanonical Pak-interactive exchange factor, cloned-out of library (PIX/COOL) binding site (84). Beyond the kinase domain (aa 529-545), the COOH-terminal end has a conserved binding site for the Gβγ-subunit of heterotrimeric G proteins, which are reported to inhibit Pak1 kinase activity (76, 125).

Significant phosphorylation sites have been identified in Pak1 for initiating and maintaining catalytic activity. Most studied is the Thr-423 site in the kinase domain, originally identified as a site of autophosphorylation following binding to GTP-bound Cdc42 or Rac1 (81, 122). This site is instrumental in sustaining relief of autoinhibition in the conformational shift from the dimerized to monomerized states and in promoting full catalytic activity toward substrates (41, 136). Although autophosphorylation at Thr-423 occurs in vitro, this activity appears to be low in vivo and instead requires phosphorylation by the 3-phosphoinositide-dependent kinase 1 in the presence of sphingosine for substantial kinase activation (64). Experimentally, substitution of the Thr-423 to glutamate renders the kinase constitutively active (81). Substitution of Lys-299 with arginine results in a kinase-dead mutant with no catalytic activity following stimulation with sphingosine or Cdc42 (16). The presence of multiple sites for phosphorylation suggest that the kinase has the capacity to interact with more than one substrate, depending on specific cellular conditions.

Structurally, inactive Pak1 exists as a homodimer with an antiparallel configuration where the COOH-terminal catalytic domain of one Pak monomer is stabilized in the inactive state by close proximity of the NH2-terminal region of the other (77) (see Fig. 2). In this trans-inhibited conformation, the NH2-terminal inhibitory switch region is associated with the C-lobe of the kinase domain of the other monomer, and the PBDs of the two monomers are associated together. Binding of GTP-bound Cdc42 to the PBD initiates dissociation of the dimer into monomers, release of the inhibitory switch from the kinase C-lobe, and exposure of the Thr-423 site for phosphorylation. Additional autophosphorylation occurs at multiple serine/threonine sites, preventing redimerization and return to an inactive state (91). The kinase-activated monomer can then interact with its cellular targets.

**Pak1 activators.** Intracellular activators of Pak1 are not limited to small GTPases but include a range of molecules. Pak1 activity may also be regulated without any involvement of protein phosphorylation. As with Cdc42/Rac1, PIX/COOL may regulate Pak1 activity in a phosphorylation-independent manner (8), although it is unclear whether PIX/COOL has any effect on autophosphorylation of Pak1. In smooth muscle cells, there is evidence for the regulation of Pak1 activity by lipid-derived signals such as sphingosine (16), lysophosphatidic acid (102), and phosphatidylinositol (64) and in COS-7 cells at a site that may be identical to the GTPase-binding domain (16). As previously discussed, receptor tyrosine kinases may activate Pak1 through interaction with Nck adapter proteins (30, 39, 112, 118). Other serine/threonine kinases phosphorylate Pak1 at sites different from the autophosphorylation sites, such as PKG, which acts at Ser-21, stimulating changes in cell morphology (38). There is recent evidence that Pak1 is activated by epidermal growth factors in a prostate carcinoma-derived cell line and that it acts through the atypical PKC-ζ isoform to phosphorylate myosin II-B, providing an important potential link between Pak1 and the cytoskeleton (36). On the side of inactivation, two protein phosphatase 2C (PP2C)-like serine-threonine phosphatases, POPX1 and POPX2, are associated with Pak1 and inactivate it through dephosphorylation, inhibiting actin stress-fiber breakdown (69, 137). In leuko-

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**Fig. 1.** Domain structure and autophosphorylation sites of p21-activated kinase 1 (Pak1). Human Pak1 has 545 amino acids. The 7 autophosphorylation sites are denoted by P. Catalytic domain (blue) contains 1 phosphorylation site (Thr-423). p21-Binding domain (PBD) is represented in orange. Autoinhibitory domain (AI) is yellow. Proline-rich motifs, represented by black bars, are followed by phosphorylation sites. These 3 motifs are between amino acids 12-18, 40-47, and 185-196, respectively. Known sites of phosphorylation by kinases are Akt (Ser-21), Cdc2/Cdk5 (Thr-212), and 3-phosphoinositide-dependent kinase 1 (Thr-423).
cytes, Pak1 activity was inhibited by pertussis toxin treatment, indicating the inhibitory G proteins function upstream of Pak1 (66). The relevance of inhibitory G proteins in the heart in antiadrenergic signaling is well established, suggesting Pak1 may be involved in some parts of this pathway. In neuronal cells, the receptor agonist acetylcholine produced the same morphological changes as do Cdc42 and Rac1 (70), suggesting that Pak1 activity is regulated by cholinergic stimulation. Stimulation of PC-12 cells and bovine brain with bradykinin (BK) induced phosphorylation of synapsin at Ser-603 that was due to Cdc42-dependent activity of Pak1 and Pak3, independent of calmodulin kinase II (CaMKII) (100). In the heart, both acetylcholine and BK are involved in ischemic preconditioning (30) (for review see, Ref. 28). The mechanism of BK-induced preconditioning may be through a reactive oxygen species signaling pathway that was blocked by inhibition of Akt (72), a kinase known to be functionally associated with Pak1. Preliminary results from our laboratory suggest that BK treatment induced dephosphorylation of troponin I (TnI) in rat ventricular myocytes concurrent with translocation of endogenous Pak1 (61). Although these individual signaling pathways in various tissues are not completely defined, many elements are consistent and also present in the heart. These agonists and pathways may also involve Pak1 signaling in the heart, suggesting potential new mechanisms underlying the functional effects.

**Pak1 targets and cellular consequences.** Pak1 is a multifunctional enzyme with numerous phosphorylation sites and binding partners that are associated with diverse cellular functions, including cytoskeletal reorganization and proliferation. The effects of Pak1 on regulation of cytoskeletal dynamics was first demonstrated in cultured mammalian cells. HeLa cells microinjected with a plasmid coding for constitutively active Pak1 had a dramatic loss of stress fibers and dissolution of focal adhesion complexes, the same effect as active forms of Cdc42 and Rac1 (81). In contrast, cells injected with a plasmid coding for active Rho showed the opposite phenotype, with increased formation of stress fibers and focal adhesions. Further evidence for Pak1 involvement in cytoskeletal signaling and interaction with growth pathways is found in a study of adhesion signaling where receptor tyrosine kinases and integrins stimulated the association of Pak1 with extracellular signal-regulated kinase (ERK) 2 in rat aortic smooth muscle. Subsequent ERK2-mediated phosphorylation of Pak1 at Thr-212 provided a negative-feedback mechanism to coordinate ERK activation by growth factor- and matrix-induced signals (112). In another study, Cdc42-activated Pak phosphorylated Lim kinase at Thr-508, increasing phosphorylation of the actin-regulatory protein cofilin (33). Mitogen-activated protein kinases (MAPKs), although generally associated with proliferative processes, may also be involved in phosphorylation of tropomyosin and thus change the activity of actomyosin. Many members of the MAPK signaling cascade were claimed to be Pak1 substrates or downstream effectors (1, 9, 31, 105, 117).

Recent findings in heart indicate localized regulation of an enzyme may be accomplished through structural proteins, which may involve Pak1-mediated signaling in processes similar to the actin-cytoskeleton regulation previously described or in the formation of enzyme complexes with localized activity. For example, A-kinase anchoring proteins (AKAPs) are necessary for the β-adrenergic-mediated phosphorylation of the L-type voltage-dependent Ca^{2+} channels [dihydropyridine receptors (DHPRs)] by PKA (40). The anchoring protein Yotiao recruits both PKA and PP1 to the slow-activating delayed rectifier potassium current (I_{Ks}) channel for the localized regulation of its phosphorylation state (23). Pakks bind to paxillin, a cytoskeletal element of focal adhesions, lending localization to cytoskeletal function (18, 48, 51). Pak1 forms a functional complex with PP2A in rat brain, creating a signaling module for specificity of dephosphorylation (129). Thus Pak1 may serve as an intermediate in localization of signaling complexes. Our laboratory has explored the possibility of similar Pak1 interactions in the heart and recently reported evidence of a Pak1-PP2A complex in adult rat ventricular myocytes (62). Expression of kinase-active Pak1 in myocytes was associated with increased PP2A activity via autodephosphorylation of the Tyr-307. Pak1 association appears to have a significant effect on PP2A localization.
activity (81, 129). Our hypothesis is that the Pak1-PP2A complex represents a novel cardiac signaling module, with Pak1 closely regulating the dephosphorylation activity of PP2A.

We propose the following scheme of Pak1-PP2A interaction, illustrated in Fig. 2. The PP2A holoenzyme, depicted in red, associates with Pak1 (blue) at its catalytic domain (53). In the inactive state the “C” subunit is phosphorylated at Tyr-307 (22). On activation of Rac1/Cdc42 by binding to GTP following stimulation of the inhibitory G protein-coupled receptor, it associates with the PBD of Pak1 and promotes the subsequent dissociation of the dimer into monomers. Pak1 autophosphorylation at Thr-423 induces a conformational change in PP2A that promotes autodephosphorylation of Tyr-307 and increased activity toward substrates. We believe that this represents a generalized mechanism for regulation of PP2A in cardiomyocytes that balances the phosphorylation processes of PKA during sympathetic stimulation and provides close control of regulatory-protein phosphorylation as well as a potential pathway for the integrated regulation of contractility at the level of both the myofilaments and Ca$^{2+}$/H$^{+}$ fluxes.

**CARDIAC CONTRACTILITY AND MORPHOLOGY: A ROLE FOR PAK1 IN INTEGRATED REGULATORY PROCESSES**

In light of the identification of a functional Pak1 and PP2A interaction in the heart, we will briefly review the contractile regulatory proteins modulated by PP2A and the structure and activation mechanisms of the phosphatase.

In the normal contractile cycle of a cardiac myocyte, the steps of electrical, chemical, and mechanical activation follow a specific sequence that is regulated at several key points (Fig. 3) [see Bers (1) and Kobayashi and Solaro (68) for reviews]. It is well known that the initial plasma membrane and t-tubule depolarization by inward voltage-dependent Na$^{+}$/H$^{+}$ channel current is rapidly followed by activation of L-type voltage-dependent Ca$^{2+}$/H$^{+}$ channels and Ca$^{2+}$ entry into the cell. This rapid increase in localized Ca$^{2+}$ in the subsarcolemmal space elicits the opening of closely apposed sarcoplasmic reticulum (SR) Ca$^{2+}$ release channels [ryanodine receptors (RyRs)] and an amplified release of Ca$^{2+}$ into the cytoplasm, forming the cytosolic Ca$^{2+}$ concentration [$\text{Ca}^{2+}$/transient. A number of associated proteins include the FK506 binding proteins (FKBP12.6) of the RyR complex (85, 133), calmodulin, CaMKII (5, 128), AKAPs (98), and the phosphatases PP1 and PP2A (12, 13). Binding of cytosolic Ca$^{2+}$ to a single site on cardiac troponin C (cTnC) strengthens the affinity of TnC for TnI and favors movement of the troponin-tropomyosin complex away from the actin-binding sites for myosin. Myosin cross-bridges then bind actin and, with sufficient ATP, cross-bridge cycling proceeds. During relaxation, Ca$^{2+}$ is released from cTnC as Ca$^{2+}$ is removed from the cytosol by resequstration to the SR through the SR Ca$^{2+}$/H$^{+}$-ATPase (SERCA) and extrusion to the extracellular space through the sodium-calcium exchanger (NCX). Thus the systolic and diastolic phases of contraction are intimately controlled by the functional state...
of DHPRs, RyRs, TnC, TnI, SERCA, phospholamban (PLB), and NCX, and likely by other proteins as well.

Because cardiac muscle must contract as a syncytium as well as meet changing metabolic demands for cardiac output, gradation of contractility is closely regulated at the cellular level. Not only the regulatory proteins themselves but also the neurohormonal signaling processes that influence phosphorylation state are significant in integrated regulation of contractility. Sympathetic activation of the Gs-coupled β1- and β2-receptors and formation of cAMP activates cAMP-dependent PKA, thus amplifying entering Ca2+ current (Ica). RyR opening, and SR Ca2+ release. PKA-dependent RyR phosphorylation likely increases channel open probability (85) and Ca2+ release. RyR opening is strongly affected by SR Ca2+ content through luminal Ca2+-sensing sites (44). Thus PKA-mediated phosphorylation of PLB at Ser-16 and the subsequent relief of SERCA inhibition permits rapid reuptake of Ca2+ to the SR, enhancing relaxation kinetics and providing additional SR Ca2+ content for the next release. Secondary to the rise in cytosolic Ca2+ concentration that accompanies PKA-mediated changes, CaMKII is activated, which further phosphorylates PLB at Thr-17 (58), leading to additional relief of SERCA inhibition, thus illustrating the complexity of phosphorylation responsiveness to agonist stimulation and the potential for interaction between the various signaling pathways. The role of NCX phosphorylation in regulating extrusion of Ca2+ from the cytoplasm into the extracellular space is controversial but does not appear to be a strong influence in mammalian ventricle (12, 127). There is evidence for phosphorylation of the Na+/-H+ exchanger, which may indirectly affect cellular Ca2+ by its influence on the levels of intracellular Na+ (106).

Myofilament protein phosphorylation in heart muscle is an additional intrinsic determinant of cardiac dynamics and contractility [see Solaro (109) and Kobayashi and Solaro (68) for reviews]. PKA-dependent phosphorylation of cardiac TnI (cTnI) at Ser-23/24 reduces the Ca2+-sensitivity of tension development, enhancing the off rate for Ca2+-exchange with cTnC (97, 126). In the physiological context of β-adrenergic stimulation and increased inotropy, the increase in available Ca2+ ensures adequate force development, and the more rapid rate of Ca2+ release from cTnC contributes to the increased lusitropy. Phosphorylation of cTnI Ser-23 and Ser-24 also increased cross-bridge cycling kinetics (63), affecting the frequency dependence of increased contraction strength and improving the speed of relaxation (114). There is evidence for phosphorylation of these sites by isoforms of PKC and by PKD (73). PKC-mediated phosphorylation of cTnI at Ser-43 and Ser-45 depresses cross-bridge kinetics as well as sensitivity to Ca2+ (93). Less is known about the Thr-144 site of cTnI, but Wang et al. (124) reported that this residue was preferentially phosphorylated by PKC-βII and was associated with an increase in myofilament Ca2+-sensitivity. PKC phosphorylation of troponin T (TnT) at Thr-206 also appears particularly functionally significant, depressing Ca2+-dependent force generation with an alteration in the ratio of tension to ATPase rate (tension cost) (111). Thick-filament phosphorylation of myosin binding protein C (MyBP-C) and of myosin light chain 2 (MLC2) regulate stretch activation of cardiac muscle and may regulate cross-bridge kinetics and the velocity of shortening (35, 99, 116, 130). All of these sites of phosphorylation are significant in the regulation of contractility at the levels of Ca2+ flux and the response of the myofilament to Ca2+. As such, they are the targets of numerous upstream signaling processes that are not completely defined, including the Pak family of kinases.

Protein phosphatases such as PP1, PP2A, and calcineurin (protein phosphatase 2B (PP2B)), are associated in functional complexes with kinases. They are also associated with intracellular Ca2+ flux and myofilament proteins, where they dephosphorylate specific regulatory sites. PP2A and PP1 both associate with PLB, where they reverse PKA-dependent phosphorylation at Ser-16. PP1 is responsible for ~60–70% of PLB dephosphorylation activity at either the Ser-16 or Thr-17 sites (80) and is inhibited both directly by PKA and through the PKA-mediated activation of inhibitor 1 (21, 34, 71, 92), further illustrating the functional interaction of kinases and phosphatas in Ca2+ flux regulation. The DHPR complex includes PP2A, where it provides localized dephosphorylation of Ser-1928 (29, 45). The RyR complex contains both PP1 and PP2A (13, 85). PP2A is the major phosphatase that dephosphorylates myofilament cTnI and MyBP-C (108), as illustrated by the okadaic acid (OA)-dependent inhibition of cTnI phosphorylation (90) and the concurrent translocation and activation of PP2A (78, 79). An additional phosphatase in the control of contractility is myosin light-chain phosphatase (MLCP), which balances the action of myosin light-chain kinase (MLCK) in control of the state of MLC2 and regulation of cross-bridge kinetics and force generation (7, 109).

PP2A structure and function. PP2A is a ubiquitous, highly regulated serine/threonine phosphatase involved in numerous cellular functions (for reviews see Refs. 27, 53, and 87). PP2A is expressed at high levels in brain and heart tissue and is involved in such diverse cellular functions as smooth muscle contraction, cell-cycle regulation, signal transduction, protein translation, apoptosis, and stress response. It is a trimeric holoenzyme composed of three subunits: a 65-kDa regulatory A subunit and a highly conserved 36-kDa catalytic C subunit that together form a central dimer, and a highly variable regulatory B subunit. The variability of the B subunit along with its targeting to specific cellular locations makes this an important element in regulating function through holoenzyme composition. Combination of a particular B subunit with the A and C core dimer serves to localize the enzyme to functional regions (88) and to confer specificity to substrates (3, 4), and as such it can affect changes in catalytic activity of a kinase toward a single substrate (55, 119). In the heart, the B56-γ regulatory subunit localizes to nuclear speckles, altering their assembly/disassembly processes, whereas the B56-α regulatory subunit was excluded from the nucleus and was found to bind the ankyrin-B adapter protein (14, 42). Disorganized expression of B56-α in reduced ankyrin-B-expressing myocytes was rescued by exogenous expression of ankyrin-B, implying it has a role in PP2A targeting.

In addition to localization, PP2A catalytic activity is regulated by posttranslational modifications such as phosphorylation and carboxymethylation. Most prominent is the decrease in activity conferred by phosphorylation of Tyr-307 in the COOH-terminal region of the catalytic C subunit. This is enhanced in the presence of OA, suggesting that PP2A autodephosphorylates at this site (22), and this is consistent with the observation that PP2A exhibits phosphotyrosyl phosphatase (PTPase) activity (2, 24). The dual-enzyme activity may be
regulated in part by the presence of a PTPase activator (54). The C subunit can also be threonine phosphorylated in vitro by an unidentified autoprophosphorylation-activated kinase, with a decrease in catalytic activity (43). The COOH-terminal motif that contains Tyr-307 has a recognition site for carboxymethyltransferase at Leu-309 by a carboxymethyltransferase (75, 134) that is reversible by the action of a specific methyltransferase (74) and may serve to stabilize the holoenzyme structure (19, 132).

Myofilament modification. Activation of Pak1 appears likely to alter the sarcomeric response to Ca\(^{2+}\) by modification of protein phosphorylation. The phosphorylation state of myofilament regulatory proteins such as cTnI, cardiac TnT (cTnT), MyBP-C, and tropomyosin are critical in regulation of contractility and are major determinants of myofilament Ca\(^{2+}\) sensitivity and cross-bridge cycling kinetics (109), but other proteins and phosphatases are active as well. Dephosphorylation of MLC2, a key regulator of myofilament Ca\(^{2+}\) sensitivity, by MLCP is well documented (7, 96). A signaling pathway involving activation of Rho kinase and regulation of the phosphatase that has been identified in smooth muscle may be of significance in heart muscle as well (46). Vahebi et al. (121) have recently elucidated a mechanism for depression of myocardial contractility by p38 MAPK. With the use of a transgenic mouse model with constitutive activation of p38 MAPK, maximum tension and ATPase activity were significantly reduced in detergent-extracted ventricular fibers concurrent with decreased phosphorylation of α-tropomyosin and cTnI. Additionally, p38 MAPK was localized at the Z disc, where it formed a complex with PP2A. These results demonstrate the presence of signaling complexes in the heart linking the activity of kinases and phosphatases, as well as establishing the integrated regulation of myofilament function and mitogenic signaling.

Another recent study of myofilament contractility through Pak signaling by Buscemi et al. (20) showed that constitutively active glutathione S-transferase (GST)-Pak3 increased the Ca\(^{2+}\) sensitivity of triton-skinned cardiac muscle fiber bundles, whereas inactive GST-Pak3 produced no change. In vitro phosphorylation of recombinant cTnI and cTnT by GST-Pak3 revealed an increase in phosphorylation of both these proteins. The site of cTnI phosphorylation was determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry to be at a novel location, Ser-149, in the inhibitory domain. Similar analysis of TnT revealed phosphorylation by GST-Pak3 within residues 68–96 and 281–290. These findings indicated a novel mechanism for direct phosphorylation of TnI by Pak3, resulting in increased myofilament Ca\(^{2+}\) sensitivity that opposes the action of PKA and PKC-mediated phosphorylation. In contrast, work done in our laboratory showed that adenovirally mediated expression of constitutively active Pak1 (AdPak1) in intact cultured adult rat ventricular myocytes induced dephosphorylation of cTnI and MyBP-C, with little change in cTnT or MLC2 phosphorylation, although Pak1 was capable of directly phosphorylating cTnI in vitro. Ca\(^{2+}\)-dependent isometric tension development in triton-extracted single myocytes that had been cultured in the presence of AdPak1 demonstrated a higher Ca\(^{2+}\) sensitivity of tension than those infected with the control virus AdLacZ, consistent with cTnI dephosphorylation.

Immunoprecipitation of expressed constitutively active Pak1 from cell lysates showed that Pak1 formed a complex with PP2A and induced dephosphorylation of the Tyr-307 residue of the PP2A catalytic subunit. This result indicates that association with active Pak1 elicits autodephosphorylation of PP2A and an increase in dephosphorylation activity toward substrates (62). Thus these studies suggest that both Pak1 and Pak3 are active in the regulation of cardiac contractility through modification of cTnI phosphorylation state. The results of Pak3 directly phosphorylating cTnI at a novel site compared with Pak1-associated activity of PP2A in dephosphorylating cTnI suggest there may be distinct signaling mechanisms for contractile regulation by specific Pak isoforms in the heart. Together, these data strongly support the hypothesis that Paks form signaling complexes in the heart that are active in cTnI regulation and have a role in reversal of the Ca\(^{2+}\)-desensitizing actions of PKA and PKC.

Ca\(^{2+}\)-flux modification. Pak1 is emerging as a potent regulator of cardiac contractility not only through PP2A-mediated dephosphorylation of cTnI and MyBP-C but also through modification of the cellular proteins that regulate Ca\(^{2+}\) fluxes. As previously discussed, the potential targets for the Pak1-PP2A signaling module in intracellular Ca\(^{2+}\) regulation include DHPRs, RyR, and PLB in control of SERCA activity (see Fig. 3). These proteins are phosphorylated by both PKA and PKC and are localized in signaling complexes with PP1, PP2A, and AKAPs (45, 57, 45, 59), but the exact signaling mechanisms for dephosphorylation have proven difficult to elucidate. PP2A is known to affect the L-type Ca\(^{2+}\) current (\(I_{\text{Ca}}\)) through a reduction in \(I_{\text{Ca}}\) and excitation-contraction (E-C) coupling gain, as reported in voltage-clamped myocytes dialyzed with PP2A. Part of the reduction in gain was thus attributable to the attenuation of \(I_{\text{Ca}}\) rather than solely to the inhibition of RyR opening (32). Alternatively, application of PP1 or PP2A to permeabilized myocytes caused a transient increase in spontaneous Ca\(^{2+}\)-spark frequency, followed by loss of spark activity and SR Ca\(^{2+}\) store depletion. Thus RyR dephosphorylation by cytosolic phosphatases resulted in increased channel activity (115). In a separate study in intact myocytes (32), intracellular dialysis with PP1 or PP2A caused a reduction in the gain of e-cc that was primarily attributed to RyR dephosphorylation. These examples illustrate the differences in the effects of PP2A found in cardiac e-cc that may be partially resolved through more complete knowledge of PP2A regulatory mechanisms.

We have recently reported (60) novel and significant evidence from a study of guinea pig sinoatrial nodal cells supporting the hypothesis that Pak1 has a role in the regulation of contractility via Ca\(^{2+}\) entry into cardiac myocytes. In this study, we demonstrated that endogenous Pak1 is abundant in these cells and that Pak1 immunoprecipitates with both the α1C-subunit of the L-type Ca\(^{2+}\) channel and with PP2A. Adenoviral-mediated expression of constitutively active Pak1 attenuated the isoproterenol-induced enhancement of \(I_{\text{Ca}}\) and the delayed-rectifier K\(^{+}\) current (\(I_{K}\)) in voltage-clamped myocytes that was reversible by PP2A inhibition. This result indicated a role for Pak1 via PP2A activity in the regulation of pacemaker activity that opposes β-adrenergic effects. The ability of Pak1 expression to blunt the isoproterenol-induced acceleration of pacemaker chronotropic effects adds evidence for the role of Pak1 in pacemaker regulation. This is the first report of Pak1-mediated effects on DHPRs and myocyte Ca\(^{2+}\) influx and contributes substantially to the emerging picture of
a Pak1-PP2A signaling complex that acts at multiple specific targets to regulate cardiac contractility.

Further evidence of Pak1 activity in Ca\(^{2+}\)-flux regulation was found through the study of the intracellular [Ca\(^{2+}\)] transient. In our laboratory, we have found evidence that the expression of constitutively active Pak1 in adult rat ventricular myocytes results in significant changes in the electrically stimulated Ca\(^{2+}\) transient compared with control as determined by fluo-4 fluorescence and line-scanning confocal microscopy (104). Myocytes expressing constitutively active Pak1 exhibited a significantly slower rate of Ca\(^{2+}\)-transient decay under basal conditions than control myocytes expressing LacZ, although the peak of the Ca\(^{2+}\) transient and unloaded cell shortening were not different. These differences continued following treatment with isoproterenol, with the exception of the significant blunting of the peak amplitude of the Ca\(^{2+}\) following treatment with isoproterenol, with the exception of the peak amplitude of the Ca\(^{2+}\) transient. Together, these data support the hypothesis of Pak1 playing a coordinating role in the regulation of cardiac contractility through the PP2A-mediated dephosphorylation of proteins controlling myofilament activity and intracellular Ca\(^{2+}\) fluxes.

Pak1 regulation of cardiac morphology. The exploration of Pak-family kinases in the heart has led to a number of important findings that build the groundwork for the significance of this kinase in the integrated regulation of contractility and morphology. An important aspect of this regulation is the integration of activity of cellular signaling cascades that modulate sarcomeric function with the pathways involved in cardiac growth and in hypertrophic responses and the transition to failure (110).

There is evidence that signaling cascades involving Pak activation also alter sarcomeric function and cell growth. For example, some upstream agonists that initiate Cdc42 and Rac1 signaling cascades are also involved in cardiac hypertrophy and dilatation. In neonatal cardiac myocytes, Rho activities increased in myocytes treated with angiotensin II, and expression of constitutively active Rho enhanced formation of stress fibers or number of sarcomeres (6, 50). Lyso phosphatidic acid, which was shown to activate Rho through inhibitory G protein in fibroblasts (120), also induced hypertrophy in neonatal cardiac myocytes (49). Kawamura and co-workers (59, 135) suggested that the effect of Rho on cytoskeletal reorganization and hypertrophy was through activation or translocation of ERK. Expression of constitutively active Cdc42 mediated by adenoviral transfer increased the ratio of length/width of the neonatal cardiac myocytes by influencing the sarcomere assembly (89). Pracyk et al. (94) reported that expression of a constitutively active Rac1 in neonatal cardiac myocytes induced sarcomeric reorganization and an increase in protein synthesis and cell size that was comparable with ligand-stimulated hypertrophy. Dominant-negative Rac1 expression attenuated the morphological changes associated with phenylephrine stimulation, establishing Rac1 as an element in cardiac hypertrophic signaling. In mouse, expression of constitutively active Rac1 by transgenesis produced two hypertrophic phenotypes, one a transient-compensated hypertrophy and one a severe dilatation. Rac1 activation was accompanied by translocation of Pak1 from the cytosol to the cytoskeleton, implying focal adhesion reorganization (113). In neonatal cardiac myocytes, MAPK signaling through the stress-activated protein kinases (SAPKs) and c-Jun N-terminal kinases (JNKs) is activated by hyperosmotic shock via treatment with sorbitol in the presence of OA. Hyperosmotic shock but neither endothelin-1 nor interleukin-1β, both agonists that stimulate SAPKs/JNKs, rapidly increased Pak1 activity, suggesting that Pak1 participates in cellular remodeling through stress-mediated pathways (26). It will be of great interest in future studies to more thoroughly investigate the role of Pak in the signaling cascades described above. This appears especially important in view of evidence that Pak1 has multiple potential effects on control of Ca\(^{2+}\) fluxes and sarcomeric response to Ca\(^{2+}\) in cardiac myocytes.

PAK1 FUNCTION IN OTHER MUSCLE TYPES

Pak1 regulation of smooth muscle contractility. Pak-family kinase function has been studied most extensively in smooth muscle, where it is involved in the phosphorylation of cytoskeletal elements involved in contraction. The activation of smooth muscle contraction differs from striated muscle, in that Ca\(^{2+}\) binds to calmodulin, inducing activation of MLCK. The action of MLCK is to phosphorylate the myosin regulatory light chain (r-MLC) at Ser-19 and Thr-18 and thereby to activate the reaction of myosin with actin, leading to ATP hydrolysis and contraction (11, 47, 56). Smooth muscle thin filaments include the regulatory protein tropomyosin but lack troponin. It is apparent that caldesmon takes the place of TnI and TnT, whereas calmodulin performs the Ca\(^{2+}\)-binding function of TnC (107). Pak1 participates in Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent contractile processes.

In a demonstration of a role for Pak in thin-filament regulation, Van Eyk et al. (123) reported that Pak induced Ca\(^{2+}\)-independent contraction of triton-skinned smooth muscle, resulting in enhanced phosphorylation of desmin and caldesmon. Although Pak phosphorylated r-MLC at Ser-19 in vitro, the direct Pak-dependent phosphorylation of r-MLC at Ser-19 has not been demonstrated in situ during contraction. MLCK is inhibited by direct Pak1 phosphorylation, which results in a decrease in phosphorylation of r-MLC and an inhibition of cell spreading in baby hamster kidney-21 and HeLa cells (101). Similarly, Pak1-mediated phosphorylation of MLCK inhibited contraction of triton-skinned guinea pig smooth muscle (131). Airway smooth muscle also exhibited GST-Pak3-mediated phosphorylation of caldesmon and desmin concurrent with an increase in force ranging from 40 to 80% (86). The GST-Pak3 phosphorylation sites in caldesmon, which were identified as Ser-657 and Ser-687, induced a depression in the binding of caldesmon to calmodulin and reduced its affinity for actin-tropomyosin (37). These findings demonstrate the importance of Pak1 in both Ca\(^{2+}\)-dependent and -independent regulation of smooth muscle contraction through specific modifications of protein phosphorylation.

CONCLUSION

There is abundant evidence for Pak1 enzyme function in the regulation of neuronal, nonmuscle cell, and muscle function, but definitions of complete pathways from agonist stimulation through activation of specific kinase sites and modification of cellular function remain unclear. Pak1 is a multifunctional enzyme that is abundant in heart, where it is active in the regulation of myocyte morphology. Pak1 also forms a signaling complex with PP2A that locally modulates the myofilament contractility.
Ca$^{2+}$ sensitivity and intracellular Ca$^{2+}$ fluxes with distinct functional effects. Although our understanding of the total function of Pak1 in the heart is currently incomplete, evidence in other cell types together with recent studies in the heart suggest that continued exploration of Pak1 function will fill in significant gaps in our knowledge of integrated contractile regulation.

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