Expression of active p21-activated kinase-1 induces Ca\(^{2+}\) flux modification with altered regulatory protein phosphorylation in cardiac myocytes

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Sheehan KA, Ke Y, Wolska BM, Solaro RJ. Expression of active p21-activated kinase-1 induces Ca\(^{2+}\)-flux modification with altered regulatory protein phosphorylation in cardiac myocytes. Am J Physiol Cell Physiol 296: C47–C58, 2009. First published October 15, 2008; doi:10.1152/ajpcell.00012.2008.—p21-Activated kinase-1 (Pak1) is a serine-threonine kinase that associates with and activates protein phosphatase 2A in adult ventricular myocytes and, thereby, induces increased Ca\(^{2+}\) sensitivity of skinned-fiber tension development mediated by dephosphorylation of myofilament proteins (Ke Y, Wang L, Pyle WG, de Tombe PP, Solaro RJ. Circ Res 94: 194–200, 2004). We test the hypothesis that activation of Pak1 also moderates cardiac contractility through regulation of intracellular Ca\(^{2+}\) fluxes. We found no difference in field-stimulated intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(i\)) transient amplitudes and extent of cell shortening between myocytes expressing constitutively active Pak1 (CA-Pak1) and controls expressing LacZ; however, time to peak shortening was significantly faster and rate of [Ca\(^{2+}\)]\(i\) decay and time of relengthening were slower. Neither caffeine-releasable sarcoplasmic reticulum (SR) Ca\(^{2+}\) content nor fractional release was different in CA-Pak1 myocytes compared with controls. Isoproterenol application revealed a significantly blunted increase in [Ca\(^{2+}\)]\(i\); transient amplitude, as well as a slowed rate of [Ca\(^{2+}\)]\(i\), decay, increased SR Ca\(^{2+}\) content, and increased cell shortening, in CA-Pak1 myocytes. We found no significant change in phospholamban phosphorylation at Ser\(^{16}\) or Thr\(^{17}\) in CA-Pak1 myocytes. Analysis of cardiac troponin I revealed a significant reduction in phosphorylated species that are primarily attributable to Ser\(^{23/24}\) in CA-Pak1 myocytes. Nonstimulated, spontaneous SR Ca\(^{2+}\) release sparks were significantly smaller in amplitude in CA-Pak1 than LacZ myocytes. Propagation of spontaneous Ca\(^{2+}\) waves resulting from SR Ca\(^{2+}\) overload was significantly slower in CA-Pak1 myocytes. Our data indicate that CA-Pak1 expression has significant effects on ventricular myocyte contractility through altered myofilament Ca\(^{2+}\) sensitivity and modification of the [Ca\(^{2+}\)]\(i\), transient.

cardiac relaxation; phosphatase; sarcoplasmic reticulum

A specialization of cardiac muscle is that contraction and relaxation dynamics rely strongly on the rapid alteration of regulatory protein phosphorylation state, much more so than in the case of skeletal muscle. These posttranslational modifications affect the sensitivity of the myofilaments to Ca\(^{2+}\) activation, cross-bridge cycling rate (15, 27), and fluxes of Ca\(^{2+}\) to and from the myofilament binding sites, thus enabling the heart to respond rapidly to changing systemic demands for cardiac output. The most prominent mechanism inducing augmentation of contraction/relaxation dynamics is β-adrenergic signaling primarily through the cAMP-dependent kinase (PKA)-mediated phosphorylation of myofilament and Ca\(^{2+}\) flux-regulatory proteins, although additional phosphorylations may occur at other sites through the action of PKC or Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CamKII).

In contrast to the involvement of kinase activity in modulation of cardiac contractility, the mechanisms signaling dephosphorylation processes in the return to basal function are not as well defined. The major cardiac phosphatases, protein phosphatase 1 and protein phosphatase 2A (PP2A), are expressed widely in the heart (38, 47) and are known to associate with regulatory proteins controlling sarcomeric function and Ca\(^{2+}\) fluxes (30, 40). Cellular agents such as adenosine and acetycholine have been identified as antiadrenergic, with associated activation of phosphatase activity (33, 48), but intermediates and localization in the signaling cascade are poorly understood. Although phosphatases such as PP2A are ubiquitously expressed, they are known to be targeted to particular locations by association with different regulatory B subunits of the holoenzyme (2, 17, 20). There is also a growing body of evidence suggesting that PP2A may be locally regulated through association with an intermediate protein (for review see Ref. 23).

In experiments reported here, we have focused on p21-activated kinase-1 (Pak1), which we recently identified as a potentially important intermediate in the PP2A signaling cascade. Pak1 is a serine-threonine kinase that is involved in actin cytoskeletal remodeling and activated by the small monomeric GTPases Rac1 and cdc42 (12, 43). Pak1 forms a complex with PP2A in neuronal cells, where it provides localization and functional specificity of the phosphatase (63). In our previous studies, we reported that endogenous Pak1 colocalizes with PP2A in cardiac myocytes at the level of the Z disk and induces its dephosphorylation at Tyr\(^{307}\), indicative of activation of the phosphatase (26). Adult rat ventricular myocytes expressing constitutively active Pak1 (CA-Pak1) exhibited a significantly reduced level of cardiac troponin I (cTnI) and myosin binding protein C phosphorylation, as assessed by incorporation of \(32^P\). This was concurrent with a leftward shift in the Ca\(^{2+}\)-tension relationship recorded in isolated detergent-extracted myocytes and a higher developed maximal tension (26). These data demonstrate that Pak1 is a novel activator of PP2A in the dynamic regulation of myofilament protein phosphorylation and contractile function of the heart. In the present study, we extend our hypothesis and propose that Pak1-mediated control of contractility goes beyond the myofilaments to include regulation of intracellular Ca\(^{2+}\) fluxes and the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(i\)) transient.

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C47
Specifically, we examined the effects of CA-Pak1 on $[\text{Ca}^{2+}]$, transients and shortening of electrically stimulated ventricular myocytes under normal and β-adrenergic-stimulated conditions. Our aim was to identify functional shifts in the intracellular $\text{Ca}^{2+}$ balance that may be associated with a phosphorylation shift of $\text{Ca}^{2+}$-regulatory proteins. Our data provide novel evidence that Pak1 catalytic activity is significant in the regulation of cardiac contractility via processes affecting $\text{Ca}^{2+}$ decay and systolic intracellular $\text{Ca}^{2+}$ fluxes, which are distinct and separable from processes regulating thin filament protein function.

METHODS

Isolation and culture of adult rat ventricular myocytes. All experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1985) and approved by the Institutional Animal Review Board of the University of Illinois at Chicago. Cardiac ventricular myocytes were isolated from the hearts of adult male 2- to 3-mo-old Sprague-Dawley rats by a collagenase perfusion method, as previously described (65). For cell culture, freshly isolated ventricular myocytes in a standard Tyrode solution [mM: 137 NaCl, 5 KCl, 10 HEPES, 1 MgCl$_2$, and 1 CaCl$_2$ (pH 7.4)] were allowed to settle onto glass coverslips (Fisher Scientific, Pittsburgh, PA) previously coated with laminin (20 µg/ml; Sigma-Aldrich, St. Louis, MO). The cells were cultured for 1 h in creative-carnitine-taurine medium (Medium 199, Cellgro, Herndon, VA) for cell attachment. The medium was changed, and the cells were cultured overnight in an atmosphere of 5% CO$_2$. Cells were infected with recombinant adenoviruses expressing CA-Pak1 or LacZ at the time the medium was changed. Expression of CA-Pak1 was determined by Western blotting of whole cell lysates with hemagglutinin (HA)-specific (Sigma-Aldrich) and Pak1-specific (Cell Signaling) antibodies (Fig. 1).

AdPak1 construction, viral amplification, and plaque assays. Using the PCR method to tag the human Pak1 cDNA with an HA epitope (YPDYVDPDYA) at the NH$_2$-terminal region and next to the transla-

tional initiation codon, we prepared recombinant adenovirus that expressed CA-Pak1 or dominant-negative kinase Pak1 (DN-Pak1). Thr$^{423}$ was mutated to glutamic acid to convert the Pak1 protein to a constitutively active form (42). The dominant-negative kinase form was made by mutation of Lys$^{290}$ to arginine (4). The cDNA was then cloned into the shuttle vector pAdCMV to obtain pAdCMVPak1. AdPak was made by homologous recombination between pAdCMVPak1 and the viral backbone DNA DL7001 (54). The lysate of virus amplified from a clone was used to infect 911 cells cultured in 100-mm dishes. The lysate from these dishes was subsequently used to infect confluent cells in five to ten 150-mm dishes. Cells were harvested at maturity, ~28–35 h after infection. The lysates were applied at the time of a discontinuous CsCl gradient and centrifuged at 20,000 g for 2 h. The lower of two bands formed during centrifugation contained mature viral particles, which were harvested using a syringe and again applied to the top of a CsCl gradient and centrifuged at 20,000 g for 12 h. The particles on the lower band were again harvested using a syringe and separated from the CsCl gradient by dialysis against HEPES-buffered saline. Virus in the dialysis unit was used directly or mixed with storage buffer and stored at $-80\degree$C. The viral titer (plaque-forming units/ml) and multiplicity of infection (MOI) were determined by standard techniques.

$[\text{Ca}^{2+}]$, imaging. Single, intact adult rat ventricular myocytes were loaded with the $\text{Ca}^{2+}$ indicator fluo 4 or fura 2 by exposure for 20 min at room temperature to a standard Tyrode solution containing 10 µM fluo 4-AM (Invitrogen, Carlsbad, CA) or 3 µM fura 2-AM (Invitrogen) made from a DMSO stock solution. Deesterification of the indicator was accomplished by a ≥15-min wash cycle in dye-free solution. Coverslips containing single myocytes were mounted on the stage of an inverted Nikon microscope and continuously perfused with a solution containing (in mM) 137 NaCl, 5 KCl, 10 HEPES, 1 MgCl$_2$, and 1 CaCl$_2$ (pH 7.4). In some experiments, 100 nM isopropyl-$\alpha$-thiogalactoside (Iso) was added to the perfusate. All experiments were performed under steady-state conditions. Single cells were field stimulated by application of a 4-ms suprathreshold square voltage pulse at 0.5 Hz to the cell bath through parallel platinum electrodes. In experiments using the confocal microscope and fluo 4, the laser scanning line was set at a central focal plane and oriented parallel to the longitudinal axis of the cell, with care taken to avoid the nucleus. The indicator was excited with the 488-nm line of an argon ion laser attached to the microscope via a confocal laser-scanning unit (Bio-Rad Radiance 2100, Carl Zeiss, Thornwood, NY) equipped with a ×60 water immersion objective. Emitted fluo 4 fluorescence was measured at >515-nm wavelengths. Images were acquired in the line-scan mode at a scanning rate of 2 ms per line. Fluorescence images were calibrated for $[\text{Ca}^{2+}]$, according to the following formula (5)

$$[\text{Ca}^{2+}] = K_R/R_0/[\text{Ca}^{2+}]_{\text{tot}} - R + 1$$

where $R$ is fluorescence (F) normalized to resting fluorescence ($F_0$; i.e., $R = F/F_0$), $K_d$ is the dissociation constant for the $\text{Ca}^{2+}$-dye complex [$K_0$ for fluo 4 was 1.1 µM (60)], and $[\text{Ca}^{2+}]_{\text{tot}}$ was 100 nM (22, 32). All experiments were performed at room temperature (i.e., 26–28°C). Acquired images were stored for later analysis using Image J (National Institutes of Health, Baltimore, MD) and Igor (WaveMetrics, Eugene, OR) software. The time constants of $[\text{Ca}^{2+}]$, transient decay ($\tau_{\text{decay}}$, ms) were evaluated by a monoexponential fit to the declining phase of the $[\text{Ca}^{2+}]$, transient.

Individual $\text{Ca}^{2+}$ spark characteristics were determined from the line-scan images with use of an automated spark detection algorithm (10). Images were processed at a level that detected release events but excluded background noise, as determined by inspection of the images. Values for amplitude ($F/F_0$), full-width at half-maximum amplitude (µm), and duration at half-maximum amplitude (ms) were tabulated from the algorithm output. $F_0$ is the fluorescence (F) recorded under steady-state conditions at the beginning of each experiment. $\text{Ca}^{2+}$ spark frequencies are expressed as the number of sparks observed per second and per 100 µm of scanned distance (sparks·s$^{-1}$·100 µm$^{-1}$).

In experiments where wide-field ($\text{Ca}^{2+}$), transients and unloaded cell shortening were recorded simultaneously, coverslips containing single myocytes loaded with fura 2 were mounted on the stage of an inverted Nikon microscope and continuously perfused with the normal Tyrode solution described above. Cells were field stimulated through parallel platinum electrodes with 4-ms suprathreshold square pulses at a frequency of 0.5 Hz. Fura 2 fluorescence and shortening of cells were recorded simultaneously, as previously described (65, 66). Fura 2-loaded myocytes were alternately excited at 340- and 380-nm wavelengths. Emitted fluorescence was measured at a wavelength of 505 nm by a photomultiplier tube at 6.67-ms intervals. Background
fluorescence was recorded daily by measurement of the autofluorescence of myocytes at both excitation wavelengths in the absence of dye loading, stored in the acquisition software (Felix 32, Photon Technology International, Birmingham, NJ), and used for subtraction in subsequent recordings. Fluorescence signals are reported as the background-subtracted ratio of fura 2 fluorescence at 340 nm to that at 380 nm (340/380 ratio). The sarcoplasmic reticulum (SR) Ca\(^{2+}\) content of the myocytes was assessed by rapid application of 10 mM caffeine to the cellular perfusate immediately after cessation of field stimulation. Field-stimulated Ca\(^{2+}\) release was expressed as a fraction of available SR Ca\(^{2+}\) according to the modified formula (1, 57)

\[
\text{fractional Ca}^{2+} \text{ release} = \frac{\text{peak twitch [Ca}^{2+}\text{]}}{\text{peak caffeine [Ca}^{2+}\text{]}}
\]

Cell shortening was recorded by illumination of the myocytes with red transmitted light (>600 nm). The cell image was collected through the microscope objective lens and transmitted to the multiimage module, where it was separated from the fluorescence signal by a 580-nm dichroic mirror. Output from the camera was split and sent to a chart recorder and to a video-edge detector (Crescent Electronics, Sandy, UT) (59). The cell length and fluorescence signals were recorded simultaneously on an acquisition computer for later analysis offline.

Preparation of myocytes for protein phosphorylation Western blotting. Freshly isolated adult rat myocytes were plated onto 60-mm tissue culture plates coated with laminin, infected with virus, and cultured overnight (see above). After the cells were treated with Iso or vehicle, the reactions were terminated with a modified radioimmunoassay precipitation buffer (RIPA, Upstate, Charlottesville, VA). SDS-PAGE was performed using a 10% polyacrylamide gel. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) and incubated overnight with the primary antibody [phosphorylated phospholamban (p-PLB; Upstate) and phosphorylated cTnI (Cell Signaling, Beverly, MA)] in Tris-buffered saline (TBS) and 5% nonfat dry milk with shaking at 4°C. The membrane was incubated in secondary antibody conjugated to horse-radish peroxidase for 1.5 h at room temperature (23°C) and developed by enhanced chemiluminescence. Films were scanned and quantified by densitometric analysis using Image J software.

Nonequilibrium isoelectric focusing. Myocytes were plated onto 60-mm tissue culture plates coated with laminin (see above), and cultured overnight after infection with CA-Pak1 or Ad-LacZ. The cultured myocytes were treated with 100 nM Iso or vehicle and harvested in TBS [in mM: 140 NaCl and 20 Tris (pH 7.4)]. Total protein content was determined using the RC DC protein assay (Bio-Rad). The isoelectric focusing gel contained 6 M urea, 5% acrylamide, 3% cross-linker (Bio-Rad), 2% Triton X-100, 0.4% ampholyte with pH 3–10, and 1.6% ampholyte with pH >9 (Amersham). Sample loading buffer contained 8 M urea, 2 M thiourea, 10 mM EDTA (pH 8.0), 1% ampholyte with pH 3–10, and 2 mM TATA binding protein. The presence of urea and EDTA facilitates the dissociation of TnI from other troponin components. The cathode buffer was placed in the upper reservoir of a Criterion gel box (10 mM H\(_2\)PO\(_4\)) and the anode buffer in the lower reservoir (100 mM NaOH), and the electrodes were reversed. The gels were run at 100 V for 20 min, 200 V for 20 min, 400 V for 20 min, and 500 V for 25 min. After isoelectric focusing, the gels were transferred to 0.2-μm nitrocellulose membranes in 10 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS) and 10% methanol (pH 11.0) at 20 V for 3 h at 0°C. Membranes were blocked with 5% nonfat dry milk in TBS-0.1% Tween 20 for 1 h at 4°C before incubation with cTnI monoclonal antibody at 1:4,000 dilution (clone C5, Fitzgerald Industries). Horse-radish peroxidase-conjugated secondary antibodies (Sigma Chemical) were used at 1:20,000 dilution, detected using ECL Plus (Pierce), and imaged using Image FX laser (Bio-Rad). Blots were quantified using Image J, and phosphorylation was expressed as follows: fraction phosphorylated = TnI (phosphorylated)/TnI (phosphorylated + unphosphorylated).

Data presentation and statistical analysis. Values are means ± SE for the indicated number of cells. Statistical significance was evaluated using Student’s t-test. In experiments where more than two groups were compared, statistical significance was evaluated using a one-way ANOVA. Statistical significance of spark frequency was evaluated using a nonparametric Mann-Whitney U test.

RESULTS

Expression of Pak1 in adult rat ventricular myocytes alters intracellular Ca\(^{2+}\) fluxes. Initially, we established the levels of CA-Pak1 protein expression in cultured adult rat ventricular myocytes. Figure 1 shows Western blots for the HA tag and for total Pak1 expression in myocytes infected with the adenoviral CA-Pak1 construct at 0, 50, 100, and 200 MOI. Expression was substantial for each MOI. All further experiments were conducted on cultured cells infected at 100 MOI. We previously established the pattern of localization of endogenous Pak1 and expressed CA-Pak1 by immunohistochemistry in rat ventricular myocytes. Endogenous Pak1 staining revealed localization in a striated pattern at the level of the Z disk, as well as localization to the cell and nuclear membranes. Adenoviral-mediated expression of CA-Pak1 showed no striations, indicating that active Pak1 has a cytosolic localization (26).

To test our hypothesis that Pak1 activity alters the regulation of contractility, in part through its action on cellular Ca\(^{2+}\) fluxes in adult rat ventricular myocytes, we used adenoviral-mediated expression of CA-Pak1 in single, isolated myocytes in combination with fluo 4 fluorescence and laser-scanning confocal microscopy to visualize the characteristics of the Ca\(^{2+}\) transient during electrical stimulation. Steady-state Ca\(^{2+}\) transients were recorded from myocytes field stimulated at 0.5 Hz before and after Iso application to reveal the effects of kinase active Pak1 expression. Figure 2 illustrates representative Ca\(^{2+}\) transients recorded from rat ventricular myocytes expressing LacZ or CA-Pak1. To construct line plots, we averaged fluorescence over the whole cell width and plotted the values vs. time. Figure 2, A and B (left), also shows the rapid, uniform rise of [Ca\(^{2+}\)]\(_i\), throughout the interior of the cells to a peak, followed by cellular shortening, [Ca\(^{2+}\)]\(_i\), decline, and relaxation. The cells were then perfused with a solution containing 100 nM Iso, and the line-scan images were again recorded when steady-state conditions were reached (Fig. 2, A and B, right). All fluorescence images were analyzed for peak [Ca\(^{2+}\)]\(_i\), and time constants of [Ca\(^{2+}\)]\(_i\) transient decay. As shown in Fig. 2C, the average [Ca\(^{2+}\)]\(_i\), transient amplitude in LacZ-expressing cells (875 ± 153 nM, n = 16 cells from 8 hearts) was not different from that in CA-Pak1-expressing myocytes (845 ± 99 nM, n = 18 cells from 8 hearts, P = 0.36). The average \(\tau_{\text{decay}}\) (Fig. 2D) was significantly slower for CA-Pak1 than for LacZ myocytes (308 ± 10 vs. 276 ± 8 ms, P = 0.02). Treatment with 100 nM Iso revealed further changes in the systolic [Ca\(^{2+}\)]\(_i\), transient characteristics. LacZ myocytes responded to Iso with a significant (P < 0.01) increase (to 1,889 ± 286 nM) in the peak [Ca\(^{2+}\)]\(_i\); the [Ca\(^{2+}\)]\(_i\) increase in CA-Pak1 myocytes was significant compared with control (non-Iso-treated) CA-Pak1 cells (1,299 ± 138 nM, P = 0.02) but was significantly less than that for Iso-treated LacZ cells (P = 0.05). The [Ca\(^{2+}\)]\(_i\) transient decay rate (\(\tau_{\text{decay}}\)) was faster in Iso-treated than in non-Iso-treated LacZ and CA-Pak1 cells but was still significantly slower in CA-Pak1 than LacZ cells (180 ± 10 vs. 149 ± 6 ms, P = 0.02). Thus the
The expression of CA-Pak1 significantly influenced the cytosolic Ca\(^{2+}\) decay rate and the capacity for peak Ca\(^{2+}\) response to Iso. In LacZ myocytes, the [Ca\(^{2+}\)]\(_i\) transient characteristics were not different from those in uninfected myocytes cultured overnight under the same conditions, which shows that expression of the viral vector did not affect the intracellular Ca\(^{2+}\) fluxes (data not shown). As a further test that the changes we observed were due to the kinase activity of CA-Pak1, we constructed DN-Pak1 virus by substituting Lys299 with arginine (4) and expressed it in cardiac myocytes by the same method used to express CA-Pak1 and LacZ. We then examined the [Ca\(^{2+}\)]\(_i\) transient parameters using field stimulation, fluo 4, and laser-scanning confocal microscopy. We found no differences in field-stimulated peak [Ca\(^{2+}\)]\(_i\) amplitude, rate of Ca\(^{2+}\) decay, levels of caffeine-releasable Ca\(^{2+}\), or fractional release between DN-Pak1- and LacZ-expressing cells. The response of DN-Pak1 myocytes to Iso was not different from that of LacZ cells (data not shown). The lack of a significant difference in [Ca\(^{2+}\)]\(_i\), transient characteristics between DN-Pak1 and LacZ cells indicates that the differences between CA-Pak1 and LacZ myocytes is due to the kinase activity induced by the Thr\(^{423}\) mutation to glutamate.

The slower rate of [Ca\(^{2+}\)]\(_i\) decay in CA-Pak1-expressing myocytes (Fig. 2) suggests that the reuptake of Ca\(^{2+}\) to the SR may be impaired, which in turn would provide less Ca\(^{2+}\) for release. To examine the SR Ca\(^{2+}\) content, we used the simultaneous measurement of wide-field fluorescence with the ratiometric Ca\(^{2+}\) indicator fura 2 and video-edge detection of unloaded cell shortening. This allowed us to evaluate the intracellular Ca\(^{2+}\) transient and, additionally, facilitated the determination of the degree and kinetics of cell shortening. All field stimulation experiments were performed at 0.5 Hz.

We found that there was no difference in the diastolic levels of fura 2 fluorescence ratio between CA-Pak1- and LacZ-expressing cells (340/380 ratio = 0.24 ± 0.02 and 0.26 ± 0.02, respectively, not significant), indicating that Pak1 activity does

![Fig. 2. Line-scan images and whole cell-width line plots of fluo 4 fluorescence recorded parallel to the longitudinal axis of the cell from representative field-stimulated adult rat ventricular myocytes expressing LacZ (blue trace, A) or CA-Pak1 (red trace, B) under control conditions and during steady-state stimulation with 100 nM isoproterenol (Iso). C: average intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) transient amplitudes in LacZ (875 ± 153 and 1,889 ± 286 nM without and with Iso, respectively, n = 16 cells from 8 hearts) and CA-Pak1 (845 ± 99 and 1,299 ± 138 nM without and with Iso, respectively, n = 18 cells from 8 hearts) cells. D: average time constant for [Ca\(^{2+}\)]\(_i\) transient decay (\(\tau_{\text{decay}}\)) for LacZ (276 ± 8 and 149 ± 6 ms without and with Iso, respectively) and CA-Pak1 (308 ± 10 and 180 ± 10 ms without and with Iso, respectively) cells. Values are means ± SE. "Statistically significant difference between LacZ and CA-Pak1.""Statistically significant difference between Iso and +Iso.](http://ajpcell.physiology.org/)
not significantly alter resting cellular Ca\(^{2+}\) under normal conditions. We tested the SR Ca\(^{2+}\) content by rapidly applying 10 mM caffeine to the cells immediately after ceasing field stimulation. Figure 3A shows that the average peak fura 2 ratio (340/380 ratio) for a normal field-stimulated twitch was 0.48 ± 0.02 in LacZ cells (n = 10 cells from 6 hearts) and 0.45 ± 0.02 in CA-Pak1 cells (n = 11 cells from 6 hearts, not significant). Perfusion of the cells with 100 nM Iso caused a significant increase in the field-stimulated LacZ Ca\(^{2+}\) peak compared with control (340/380 ratio = 0.71 ± 0.05, P < 0.01). In CA-Pak1 cells, Iso also increased the field-stimulated peak Ca\(^{2+}\) compared with control (340/380 ratio = 0.58 ± 0.03, P < 0.05), but this value was significantly blunted compared with the LacZ value (P = 0.01), confirming the fluo 4 results.

The caffeine-induced peaks (Fig. 3B) were not significantly different in LacZ- and CA-Pak1-expressing cells (340/380 ratio = 0.63 ± 0.02 and 0.53 ± 0.04, respectively, not significant); however, there was a trend toward a lower level of SR Ca\(^{2+}\) content in CA-Pak1 myocytes (P = 0.09). The caffeine-releasable SR Ca\(^{2+}\) contents of each expression type increased with Iso treatment compared with its own control but were not different from each other (340/380 ratio = 0.78 ± 0.04 and 0.69 ± 0.06 for LacZ and CA-Pak1, respectively, not significant). The fractional SR Ca\(^{2+}\) release, an indicator of the amount of available SR Ca\(^{2+}\) content that is released during a normal field-stimulated twitch contraction, was calculated as follows: (peak field-stimulated [Ca\(^{2+}\)]/peak [Ca\(^{2+}\)], elicited by caffeine) × 100. Figure 3C shows no difference in the fractional release between LacZ and CA-Pak1 cells (76 ± 3% and 87 ± 3%, respectively). After treatment with Iso, the fractional release increased significantly only in LacZ cells compared with control (91 ± 5%, P < 0.05). The fractional release in CA-Pak1 cells was not different from control (85 ± 3%, not significant), and there was no difference between LacZ and CA-Pak1 cells. These results suggest that the blunting of the Ca\(^{2+}\) release response to Iso in CA-Pak1 cells is in part due to the effects of the kinase on the excitation-contraction coupling release mechanism, rather than reduction of the SR Ca\(^{2+}\) available for release.

The simultaneous recordings of field-stimulated fura 2 fluorescence and cellular shortening allowed us to more closely examine the effects of CA-Pak1 expression on the contractile characteristics of intact myocytes and their relationship to the [Ca\(^{2+}\)] transient. Figure 4A illustrates a typical field-stimulated contraction of a LacZ-expressing cell, along with the associated [Ca\(^{2+}\)] transient before and after treatment with Iso. Cell shortening was normalized to total cell length for clarity of presentation. Iso application caused an increase in the amplitude of cell shortening for LacZ cells, on average from 4.1 ± 0.9% to 12.3 ± 1.4% (P < 0.01), corresponding to the fluorescence (340/380 ratio) increase from 0.48 ± 0.03 to 0.71 ± 0.05 (P < 0.01). A typical recording under the same conditions in a CA-Pak1 cell is shown in Fig. 4B. For CA-Pak1 cells, the average cell shortening increased from 6.4 ± 1.0% to 12.2 ± 2.2% in the presence of Iso (P < 0.01), in conjunction with the fluorescence (340/380 ratio) increase from 0.45 ± 0.02 to 0.58 ± 0.04 (P < 0.05). Although the increase in shortening in the presence and absence of Iso was significant for LacZ and CA-Pak1 myocytes, the relationship between the peak activating Ca\(^{2+}\) and percent shortening was significantly changed in the presence of Iso. Figure 4C shows the average peak shortening as a function of fura 2 fluorescence for LacZ and CA-Pak1 cells. In the absence of any treatment, there was no difference in the degree of shortening for the available Ca\(^{2+}\)
In LacZ or CA-Pak1 cells, although the CA-Pak1 cells shortened slightly more at a slightly lower [Ca\textsuperscript{2+}]. With Iso treatment, however, shortening was virtually the same for both types of cells, with a significantly lower peak [Ca\textsuperscript{2+}] in CA-Pak1 myocytes. This clear shift in the Ca\textsuperscript{2+} sensitivity of shortening confirms our previous finding of increased Ca\textsuperscript{2+} sensitivity of the myofilaments (26). Figure 4D summarizes the time to peak shortening for LacZ and CA-Pak1 myocytes without and with Iso. The time to peak shortening was significantly faster in CA-Pak1 than LacZ myocytes (140.0 ± 11.3 ms vs. 172.1 ± 10.2 ms, P < 0.05). Application of Iso significantly reduced the time to peak contraction for LacZ and CA-Pak1 cells (119.1 ± 4.1 and 93.5 ± 7.7 ms respectively) compared with controls, but the difference between them did not reach significance. These data demonstrate that Pak1 kinase activity modifies the processes regulating intracellular Ca\textsuperscript{2+} fluxes independently of its effects on the myofilament. These distinct, separable effects of Pak1 on the two major processes regulating beat-to-beat contractile strength are evidence of the dual nature of Pak1 in the integrated regulation of cardiac contractility.

To further evaluate the effects of CA-Pak1 on intracellular Ca\textsuperscript{2+}, we used confocal microscopy to examine Ca\textsuperscript{2+} sparks. In cardiac myocytes, the electrically stimulated [Ca\textsuperscript{2+}] transient is composed of elementary events of SR Ca\textsuperscript{2+} release, or Ca\textsuperscript{2+} sparks (9, 36), that represent the opening of clusters of the SR Ca\textsuperscript{2+} release channels, or ryanodine receptors (RyRs). These openings may be spontaneous, particularly in the case of...
SR Ca\(^{2+}\) overload, or they may be triggered by unitary Ca\(^{2+}\) current through voltage-dependent L-type Ca\(^{2+}\) channels, or dihydropyridine receptors (DHPRs) (35). Figure 5A shows typical line-scan images illustrating Ca\(^{2+}\) sparks in single intact, quiescent LacZ- and CA-Pak1-expressing myocytes loaded with the Ca\(^{2+}\)-indicator fluo 4 under Ca\(^{2+}\)-overload conditions (2 mM Ca\(^{2+}\) perfusion solution). The laser scanning line was set at a central focal depth parallel to the longitudinal axis of each cell. We found no difference in the frequency of spark occurrence between the two groups [1.25 ± 0.4 (n = 10 cells) and 1.92 ± 0.45 sparks·s\(^{-1}\)·100 μm\(^{-1}\) (n = 18 cells) for LacZ and CA-Pak1, respectively, not significant]. As shown in Fig. 5B, there was a significant reduction in spark amplitude in CA-Pak1 (3.05 ± 0.04 F/F\( _{0} \), n = 214 sparks) compared with LacZ (3.28 ± 0.09 F/F\( _{0} \), n = 73 sparks) cells (P < 0.01) and in spatial spread (1.08 ± 0.07 and 1.45 ± 0.17 μm in CA-Pak1 and LacZ, respectively, P < 0.01). The temporal durations were not different (22 ± 3 and 19 ± 1 ms for LacZ and CA-Pak1, respectively, not significant).

Under Ca\(^{2+}\)-overload conditions, sparks can initiate additional Ca\(^{2+}\) release at adjacent sites in the form of propagating Ca\(^{2+}\) sparks or spontaneously propagating Ca\(^{2+}\) waves (8). LacZ- and CA-Pak1-expressing myocytes exhibited propagation of sparks under overload conditions and fusion of sparks into waves. Line-scan confocal images in Fig. 5C illustrate spontaneous propagating release from each type of cell bathed in 2 mM extracellular Ca\(^{2+}\). The line plots were constructed by averaging fluo 4 fluorescence over 10-μm regions of interest indicated on the edge of the image. The laser scanning line was set parallel to the longitudinal axis of each cell, so the regions of interest show the characteristics of the wave at each end of the myocyte. The average amplitudes of release were not different [1.147 ± 190 nM in LacZ (n = 8 cells from 4 hearts) and 1.041 ± 108 nM in CA-Pak1 (n = 10 cells from 3 hearts)], but the kinetics of the Ca\(^{2+}\) release were altered in CA-Pak1 myocytes. The upstroke of Ca\(^{2+}\) was significantly slower in CA-Pak1 than LacZ cells (275 ± 22 vs. 167 ± 23 ms, P < 0.01). The time constant of [Ca\(^{2+}\)]\( _{i} \) decay was also slower in CA-Pak1 than LacZ cells (527 ± 30 vs. 399 ± 48 ms, P < 0.05). Finally, the velocity of propagation was significantly slower in CA-Pak1 than LacZ cells (31 ± 2 vs. 52 ± 6 μm/s, P < 0.01). The presence of Ca\(^{2+}\) sparks in CA-Pak1 cells at the same frequency as in LacZ cells, but with a lower amplitude and spatial spread, suggests an alteration of RyR function within the SR Ca\(^{2+}\) release units. The slower upstroke and decay of Ca\(^{2+}\) waves, along with the slower velocity of propagation, suggest an impaired ability for cytosolic Ca\(^{2+}\) to activate neighboring release units.

We used Western blotting to determine the phosphorylation states of specific regulatory proteins involved in mediating

![Fig. 5. A: spontaneous Ca\(^{2+}\) sparks were visualized in LacZ- and CA-Pak1-expressing myocytes using fluo 4 fluorescence (top) and laser-scanning confocal microscopy in the line-scan mode (bottom) at a central location in the cell. Line plots were constructed by averaging fluorescence over 10-μm regions of the cell length (vertical line at left). B: in quiescent myocytes under Ca\(^{2+}\)-overload conditions (2 mM extracellular Ca\(^{2+}\)), average LacZ spark amplitude was significantly (P < 0.01) larger for LacZ than CA-Pak1 cells: 3.28 ± 0.09 F/F\( _{0} \) (n = 10 cells from 5 hearts) vs. 3.05 ± 0.04 F/F\( _{0} \) (n = 18 cells from 4 hearts). Full width at half-maximum amplitude (FWHM) was also larger for LacZ than CA-Pak1 myocytes (1.5 ± 0.2 vs. 1.1 ± 0.1 μm, P < 0.01). Temporal durations were not different (22 ± 3 and 19 ± 1 ms for LacZ and CA-Pak1, respectively, not significant). C: line-scan images of fluo 4 fluorescence. Note spontaneous waves of SR Ca\(^{2+}\) release in quiescent myocytes under Ca\(^{2+}\)-overload conditions (2 mM extracellular Ca\(^{2+}\)). Values are means ± SE. *Statistically significant difference.](http://ajpcell.physiology.org/)

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intracellular Ca\(^{2+}\) fluxes and myofilament function. Phosphorylation of PLB relieves inhibition of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase activity and speeds the reuptake of cytosolic Ca\(^{2+}\) to the SR (31); thus the slowed rate of \([\text{Ca}^{2+}]_c\) decay we observed may be attributable to PLB dephosphorylation. Figure 6 shows a representative Western blot of LacZ and CA-Pak1 myocyte lysates probed for PLB phosphorylated at Ser\(^{16}\) and Thr\(^{17}\) and total PLB (A1 clone). LacZ and CA-Pak1 cells had relatively low levels of Ser\(^{16}\) phosphorylation (5.2 ± 2\% and 5.3 ± 2\% of total, \(n = 5\) hearts, not significant; Fig. 6B). After treatment with 100 nM Iso, PLB phosphorylation at Ser\(^{16}\) was not significantly different between LacZ and CA-Pak1 cells (100 ± 7 and 97 ± 9\%, not significant; Fig. 6B). Levels of Thr\(^{17}\) phosphorylation were not significantly different without Iso treatment (23 ± 6\% and 27 ± 6\% in LacZ and CA-Pak1, respectively) or after Iso treatment (71 ± 4\% and 80 ± 5\% in LacZ and CA-Pak1, respectively). The lack of a significant shift in PLB dephosphorylation in CA-Pak1 myocytes, particularly at Ser\(^{16}\) in the presence of Iso, indicates that participation of Pak1 in dephosphorylation activity at this site is not sufficient to oppose the PKA-mediated increase in PLB phosphorylation and, thus, SR Ca\(^{2+}\) uptake.

We used a novel nonequilibrium isoelectric focusing method developed in our laboratory (28, 56) to evaluate the cTnI phosphorylation states in LacZ and CA-Pak1 myocytes. This method has the advantage that the phosphorylation species of cTnI can be separated into bands representing the unphosphorylated protein as well as several additional phosphorylated sites. The phosphorylation states represented by each band are additive, as previously shown by Kobayashi et al. (28) and Scruggs et al. (56), with each higher-migrating band also containing phosphorylated species from the lower bands. A representative blot of LacZ and CA-Pak1 whole cell lysates (Fig. 7A) illustrates the phosphorylation profile of cTnI without and with 100 nM Iso. We observed the same migration pattern of at least five distinct phosphorylated species of cTnI for LacZ and CA-Pak1. The lowest band, U, is the unphosphorylated cTnI protein. The next-higher band, P2, contains species phosphorylated at the Ser\(^{23/24}\) sites, whereas the P4 band consists of species phosphorylated at other sites in addition to Ser\(^{23/24}\). The U bands were most prominent for LacZ and CA-Pak1 cells, but LacZ and CA-Pak1 cells had bands in the positions for the P2, P4, and P6 species. After treatment with Iso, the U bands were diminished and the P2 and P4 bands were more intense. The LacZ and CA-Pak1 cell lysate U band densities were not different (56 ± 6\% and 60 ± 4\% for LacZ and CA-Pak1, respectively, \(n = 4\) hearts for all results, not significant; Fig. 7B), nor were the Ser\(^{23/24}\)-containing bands (23 ± 3\% and 27 ± 2\% (P2) for LacZ and CA-Pak1, respectively, not significant; 22 ± 4\% and 14 ± 5\% (P4) for LacZ and CA-Pak1, respectively, not significant). Iso treatment decreased the levels of unphosphorylated cTnI in LacZ and CA-Pak1 cells (20 ± 5\% and 19 ± 5\%, not significant). The P2 band of Ser\(^{23/24}\) phosphorylation was significantly lower in CA-Pak1 than LacZ cells (28 ± 3\% vs. 36 ± 1\%, \(P = 0.04\)). The P4 bands also were not different between LacZ and CA-Pak1 cells stimulated with Iso (44 ± 5\% and 53 ± 8\%, not significant). These results confirm our previous finding that CA-Pak1 expression in myocytes results in a functional reduction in the Ser\(^{23/24}\) phosphorylation of cTnI.

**DISCUSSION**

Our data present novel evidence that CA-Pak1 expressed in single, isolated adult rat ventricular myocytes significantly changes the decay and peak characteristics of the \([\text{Ca}^{2+}]_c\) transient and that the peak changes are more pronounced after stimulation with Iso. We further establish that this shift in the processes controlling the delivery of Ca\(^{2+}\) to the myofilaments is distinct from the changes in the myofilament contractile response.

In general, the mechanisms that modulate the phosphorylation state of cardiac contractile regulatory proteins depend on the dynamic balance of kinase activity with cellular phosphatase activity, although the dephosphorylation control mechanisms are not well understood (16, 29, 58, 62). Pak1 has been identified as a component of a functional protein complex, along with PP2A in neuronal cells, where it is significant in the localized regulation of PP2A activity (63). Previous evidence from this laboratory has established that CA-Pak1 expressed in adult rat ventricular myocytes is localized at the Z disk and facilitates the autodephosphorylation of PP2A at Tyr\(^{587}\) (26),signifying increased catalytic activity (7). Functionally, CA-
After treatment with Iso, the slower rate of \([\text{Ca}^{2+}]_i\), transient decay attributable to CA-Pak1 expression remained evident. Along with the absence of a significant change in PLB phosphorylation relative to LacZ, this again indicates that CA-Pak1 influences the \([\text{Ca}^{2+}]_i\) decay rate independently of PLB. Additionally, the expected increase in the \([\text{Ca}^{2+}]_i\) transient peak amplitude was significantly blunted in CA-Pak1 compared with LacZ cells. The SR \([\text{Ca}^{2+}]_i\) content increased significantly with Iso for LacZ and CA-Pak1 cells, with no change in the fractional release, indicating that the blunted peak \([\text{Ca}^{2+}]_i\) in CA-Pak1 cells was not due to a lack of available \([\text{Ca}^{2+}]_i\). Alternatively, this indicates an alteration in the excitation-contraction coupling mechanism.

Pak1 appears to have multiple roles as part of a PP2A signaling complex and, therefore, potentially can act at numerous target proteins. PP2A has been identified as a part of the RyR2 SR \([\text{Ca}^{2+}]_i\) release channel complex, along with FK-binding protein 12.6, PKA, PP1, and muscle-specific A-kinase anchoring protein (44, 46). The mechanisms of RyR2 functional regulation, particularly with regard to the identification of phosphorylation sites and their functional consequences, are controversial. Marx et al. (46) and Valivia et al. (61) showed that PKA phosphorylates RyR2 at Ser2809 and that PKA-mediated phosphorylation increased the channel peak open probability (\(P_o\)). Hyperphosphorylation of RyR2 by PKA, particularly in failing human hearts, appears to cause dissociation of FK-binding protein 12.6 from the RyR2 channel, leading to persistent gating and SR \([\text{Ca}^{2+}]_i\) leak (45, 46). Recent evidence, however, suggests that adrenergic stimulation and PKA effects on contractility are not mediated through the Ser2808 site of RyR2 (39). Elementary events of SR \([\text{Ca}^{2+}]_i\) release, or \([\text{Ca}^{2+}]_i\) sparks, result from the opening of a cluster of RyRs (3) and summate to form the intracellular \([\text{Ca}^{2+}]_i\) release transient (6, 9). We thus examined spontaneous \([\text{Ca}^{2+}]_i\) sparks in LacZ and CA-Pak1 myocytes to gain insight into RyR function. The frequency and amplitude of \([\text{Ca}^{2+}]_i\) sparks are determined in part by the SR \([\text{Ca}^{2+}]_i\) content (8, 37, 55). We observed a significant reduction in the amplitude of spontaneous \([\text{Ca}^{2+}]_i\) sparks in CA-Pak1 compared with LacZ cells but no change in the frequency of occurrence. The lower amplitude may be in part due to the slight decrease in SR \([\text{Ca}^{2+}]_i\) content we observed (Fig. 3), but we would also expect the spark frequency to be lower under these conditions. Alternatively, the lower spark amplitude in CA-Pak1 myocytes may suggest that the RyR channel \(P_o\) may be lower, so that fewer RyRs are gating within a cluster. The spatial spread of the sparks was reduced, but the temporal duration was unchanged, suggesting that fewer channels opened but the kinetics of opening and closing were the same. The slower propagation velocity of spontaneous \([\text{Ca}^{2+}]_i\) waves indicates a resistance to activation of adjacent RyR clusters by cytosolic \([\text{Ca}^{2+}]_i\) in CA-Pak1 cells. Thus Pak1 activity may alter RyR2 gating via dephosphorylation by PP2A, but a full characterization is beyond the scope of this work.

Another potential target for Pak1 activity in the excitation-contraction coupling mechanism is the voltage-dependent L-type \([\text{Ca}^{2+}]_i\) channel, or DHPR. This sarcosomal ion channel forms a functional complex with a number of kinases, phosphatases, and anchoring proteins (for review see Ref. 24), including PKA and PP2A. Phosphorylation by PKA during adrenergic stimulation increases L-type \([\text{Ca}^{2+}]_i\) channel opening, providing additional \([\text{Ca}^{2+}]_i\) entry to trigger SR \([\text{Ca}^{2+}]_i\) release.

Pak1 expression induced dephosphorylation of cTnI and myosin binding protein C, with a concurrent increase in the \([\text{Ca}^{2+}]_i\) sensitivity of tension development in detergent-extracted myocytes. The myofilament response and also the cellular \([\text{Ca}^{2+}]_i\) fluxes that supply activating \([\text{Ca}^{2+}]_i\) to the myofilaments are modulated by regulatory protein phosphorylation state and, thus, alter contractility. This leads to our present hypothesis that Pak1 catalytic activity regulates cardiac contractility through the integrated modification of intracellular \([\text{Ca}^{2+}]_i\) fluxes, as well as myofilament \([\text{Ca}^{2+}]_i\) sensitivity.

We observed a change in the \([\text{Ca}^{2+}]_i\) characteristics consisting of a significantly slower rate of \([\text{Ca}^{2+}]_i\) decline in CA-Pak1 than LacZ-expressing controls, concurrent with longer time for cellular relengthening. The levels of PLB phosphorylation at Ser16 and Thr17 were not significantly different between CA-Pak1 and control myocytes, nor was cTnI phosphorylation at Ser23/24 significantly different. There was no change in CA-Pak1 cell SR \([\text{Ca}^{2+}]_i\) content as assessed by caffeine, consistent with the lack of change in PLB phosphorylation. This indicates CA-Pak1 slows the intracellular \([\text{Ca}^{2+}]_i\) decay rate independently of PLB, although the exact mechanism remains to be determined.
PKA reduces both L-type Ca\(^{2+}\) current (\(I_{Ca}\)) and the intracellular Ca\(^{2+}\) transient (14), although the activating mechanism of the phosphatase is unclear. A recent study from our laboratory (25) established the association of Pak1 with PK2A in guinea pig pacemaker cells, where it inhibited \(I_{Ca}\) and the delayed rectifier K\(^+\) current after stimulation with Iso. Reduction of \(I_{Ca}\) by PK2A via Pak1 activation could account for the blunting of the electrically stimulated transient under Iso stimulation. Thus Pak1 may have a role in altered DHPR function in ventricular myocytes, as well as in pacemaker cells.

The simultaneous recording of unloaded cell shortening and fura 2 fluorescence enabled us to correlate the CA-Pak1-induced changes in the [Ca\(^{2+}\)]\(_i\) transient with changes in the shortening characteristics. We found that the CA-Pak1 myocytes exhibit a greater Ca\(^{2+}\) sensitivity of contraction (Fig. 4C) as a result of myofilament modification, and, at the same time, the [Ca\(^{2+}\)]\(_i\) transient is reduced because of changes in the excitation-contraction coupling process. We also showed a significantly faster time to peak shortening and slower time to 90% relengthening in CA-Pak1 myocytes. Although this difference did not reach significance in the presence of Iso, there was a trend toward a faster time of shortening and slower relengthening, which supports our hypothesis that Pak1 activity modifies excitation-contraction coupling separately from myofilament contractility.

In the present study, we have focused on PKA-mediated regulatory protein phosphorylation sites, but other groups of kinase pathways in the heart potentially involve Pak1. When activated by Ca\(^{2+}\)-bound calmodulin, CaMKII phosphorylates a number of excitation-contraction coupling-related proteins (for review see Ref. 40), including RyR2 at Ser2809. The same site as PKA, but it is uncertain whether this results in a higher or lower channel \(P_a\) (33, 62). \(I_{Ca}\) is facilitated by CaMKII phosphorylation (18). PLB is phosphorylated at Thr\(^{17}\) by CaMKII and plays a role in the regulation of frequency-dependent acceleration of relaxation (FDAR) (52), although other CaMKII-related processes appear to be involved (12). There is recent evidence that CaMKII activity in FDAR may be influenced by kinase autophosphorylation and the activity of associated protein phosphatases (10). Since Pak1 is known to associate with PP2A and modify its activity, there may be a role for Pak in FDAR. In the present study, we have shown that CA-Pak1 expression does not significantly alter the levels of Thr\(^{17}\) phosphorylation of PLB, but time of relengthening is slower in CA-Pak1 than LacZ cells. The ability of CA-Pak1 to change relaxation rates suggests that it may have a further role in the regulation of FDAR.

The PKC family of kinases is also significant in the regulation of contractility, primarily through phosphorylation of myofilament regulatory proteins (17, 20, 48, 50, 51), modifying the Ca\(^{2+}\) sensitivity of force, and the cross-bridge cycling rate. Previous studies have indicated that cTnI can be phosphorylated at Ser\(^{23/24}\) by PKC\(\beta\), PKC\(\epsilon\) (27), and PKC\(\delta\) (49) in vitro, thus participating in the regulation of myofilament function. A recent study from our laboratory has indicated that PKC\(\gamma\) forms a complex with Pak1 and PP2A, resulting in reduced threonine phosphorylation of cTnI and cTnT (65). Thus Pak1 involvement in contractile regulation of the thin filament potentially involves PKC-mediated phosphorylation processes.

Our data provide significant novel evidence for the functional action of Pak1 in the integrated regulation of cardiac contractility through modification of intracellular Ca\(^{2+}\) fluxes in intact ventricular myocytes, distinct from its effects on thin filament regulation. We propose that Pak1 is an important novel regulator of heart function that remains to be fully explored.

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