Receptor-Independent Cardiac Protein Kinase Cα Activation by Calpain-Mediated Truncation of Regulatory Domains

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Rationale: Protein kinase (PK)Cs and calpain cysteine proteases are highly expressed in myocardium. Ischemia produces calcium overload that activates calpains and conventional PKCs. However, calpains can proteolytically process PKCs, and the potential in vivo consequences of this interaction are unknown.

Objective: To determine the biochemical and pathophysiological consequences of calpain-mediated cardiac PKCα proteolysis.

Methods and Results: Isolated mouse hearts subjected to global ischemia/reperfusion demonstrated cleavage of PKCα. Calpain 1 overexpression was not sufficient to produce PKCα cleavage in normal hearts, but ischemia-induced myocardial PKCα cleavage and myocardial injury were greatly increased by cardiac-specific expression of calpain 1. In contrast, calpain 1 gene ablation or inhibition with calpastatin prevented ischemia/reperfusion induced PKCα cleavage; infarct size was decreased and ventricular function enhanced in infarcted calpain 1 knockout hearts. To determine consequences of PKCα fragmentation on myocardial protein phosphorylation, transgenic mice were created conditionally expressing full-length PKCα or its N-terminal and C-terminal calpain 1 cleavage fragments. Two-dimensional mapping of ventricular protein extracts showed a distinct PKCα phosphorylation profile that was exaggerated and distorted in hearts expressing the PKCα C-terminal fragment. MALDI mass spectroscopy revealed hyperphosphorylation of myosin-binding protein C and phosphorylation of atypical substrates by the PKCα C-terminal fragment. Expression of parent PKCα produced a mild cardiomyopathy, whereas myocardial expression of the C-terminal PKCα fragment induced a disproportionately severe, rapidly lethal cardiomyopathy.

Conclusions: Proteolytic processing of PKCα by calcium-activated calpain activates pathological cardiac signaling through generation of an unregulated and/or mistargeted kinase. Production of the PKCα C-terminal fragment in ischemic hearts occurs via a receptor-independent mechanism. (Circ Res. 2010;107:903-912.)

Key Words: protein kinase C ■ calpain 1 ■ cardiomyopathy ■ ischemia/reperfusion injury ■ myocardial infarction ■ myosin-binding protein C

Receptor-mediated activation of protein kinase (PK)Cs via Gαq and phospholipase C signaling is implicated in myocardial contractile abnormalities, cardiac hypertrophy, and the progression to heart failure.1,2 Of the 12 recognized PKC isoenzymes,3 myocardium expresses significant amounts of 4: 2 “conventional” isoenzymes (PKCα and PKCβ) and 2 “novel” isoenzymes (PKCδ and PKCe). PKCα is the most abundant PKC in mouse myocardium and is specifically implicated in cardiac hypertrophy, contractile dysfunction, and cardiomyopathy development.4–8

The canonical mechanism for PKC activation is via phospholipase C–mediated increases in cytosolic free calcium and phospholipids. Walsh and colleagues have suggested that oxidative stress can stimulate a pathway to PKC activation that is independent of these receptor-coupled signaling events, although specific mechanisms were not identified.9 PKCs consist of an N-terminal regulatory domain connected to a C-terminal catalytic domain via an interdomain V3 “hinge” region.10 In their inactive state, the N-terminal regulatory and C-terminal catalytic domains interact to sequester critical membrane targeting and substrate-binding domains. Conventional PKC isoenzymes, including PKCα, are activated when calcium and phospholipid bind to specific domains within the N terminus and disrupt intramolecular bonding, thus unfolding the molecule and exposing C-terminal substrate binding domains.11 Conventional wisdom holds that concomitant with PKC activation, calcium and phospholipid initiate PKC inactivation through calpain-mediated proteolysis.12–14

Calpains are ubiquitous calcium-dependent cysteine proteases.15 We previously detected low levels of calpain 1, but not calpain 2, proteolytic activity in nonstressed myocardium.16 However, myocardial free calcium levels rise dramatically to

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600 to 1000 nmol/L (depending on perfusate calcium concentration) during ischemia/reperfusion, exceeding levels that induce calpain activation in cardiac myocytes. For this reason, calpain-mediated degradation of myofilament proteins has been implicated in postischemic myocardial injury and stunning and in preload-induced proteolysis. Calpain proteolysis of myocardial PKCα has not been examined. Here, we show that calpain 1 cleaves myocardial PKCα as a specific response to ischemia/reperfusion. We demonstrate that the C-terminal PKCα cleavage fragment is a potent myocardial kinase. Finally, we determine that the PKCo C-terminal fragment, but not the N-terminal fragment, induces cardiomyopathic features, independent of myocardial ischemia or calpain activation.

Methods

Transgenic Mice

Mice conditionally overexpressing calpain 1 with a tetracycline-suppressible (“tet-off”) α-mycosin heavy chain–driven conditional transgenic system have been described previously. The present studies used the lower-expressing “L2” line with calpain induction at 8 weeks of age. This line of calpain 1 transgenic mice does not exhibit spontaneous proteolysis and does not develop a cardiac phenotype after transgene induction in adult hearts. Calpain 1 knockout mice were previously described.

Human PKCα and fragments encoding peptides corresponding to the calpain N-terminal cleavage fragment (PKCαNT) and C-terminal cleavage fragment (PKCoCT) were expressed using the same conditional cardiac-specific expression system. Invasive hemodynamic studies and M-mode echocardiography were performed using a time- and concentration-dependent approach. We demonstrated that PKCα cleavage product after 1 hour of reperfusion in normal hearts, but not calpain 1 knockout hearts (Figure 1A). This result suggested that PKCα is a substrate for proteolysis by calpains activated after myocardial ischemia. Accordingly, we performed in vitro studies to define the products of calpain 1–mediated PKCα proteolysis. Calpain 1 cleaved PKCα into two 40-kDa fragments in a manner that was time- and concentration-dependent (Figure 1B, top and lower left). Ex vivo proteolysis of myocardial PKCα required addition of calcium (Figure 1B, lower right).

We took advantage of previously described cardiac transgenic mice expressing calpain 1 or the calpain inhibitor protein calpastatin (CSTN), to examine the consequences of cardiomyocyte calpain 1 activation or inhibition on PKCα fragmentation in ischemic hearts. The 40-kDa PKCα fragment was detected only in ischemia-reperfused calpain 1 hearts (Figure 1C, left); characteristic calpain-mediated proteolysis of troponin I and T was also observed (Figure 1C, left). No such fragmentation was detected in identically treated CSTN hearts (Figure 1C, right). Thus, ischemia/reperfusion induces calpain 1–mediated fragmentation of myocardial PKCα.

Statistical Methods

All results are presented as means±SEM. Paired group analysis used Student’s t test. Multiple groups were compared by one-way ANOVA and Tukey’s post hoc test. P<0.05 was considered significant.

Results

Ischemia Stimulates Calpain 1–Mediated Limited Proteolysis of Myocardial PKCα

In the process of assessing PKC activation by cardiac ischemia/reperfusion, we observed formation of a single ~40-kDa PKCα cleavage product after 1 hour of reperfusion in normal hearts, but not calpain 1 knockout hearts (Figure 1A). This result suggested that PKCα is a substrate for proteolysis by calpains activated after myocardial ischemia. Accordingly, we performed in vitro studies to define the products of calpain 1–mediated PKCα proteolysis. Calpain 1 cleaved PKCα into two ~40-kDa fragments in a manner that was time- and concentration-dependent (Figure 1B, top and lower left). Ex vivo proteolysis of myocardial PKCα required addition of calcium (Figure 1B, lower right).
Calpain 1 Increases Postinfarction Myocardial Damage

To determine the functional consequences of calpain activation in ischemic myocardium, we measured infarct size and outcomes after coronary ligation of conditional calpain 1 transgenic (FVB/N background) and calpain knockout mice (C57 background). (Calpastatin transgenic mice develop an aggresomal cardiomyopathy that confounds infarction studies.14) Infant size at 24 hours was increased by approximately one-third in calpain 1–expressing mice (Figure 2A), and early survival was strikingly decreased (Figure 2B). We used fluorescent TUNEL staining to assess cardiac myocyte death in the noninfarct myocardium, which was increased ≈50% (Figure 2C).

The reciprocal experiment with calpain 1 knockout mice gave the opposite result. Although strain differences resulted in different absolute infarct sizes between the overexpression and knockout studies, calpain 1 knockout mice showed no difference in postinfarction mortality (not shown), had a modest reduction in infarct size measured 4 days after coronary occlusion (Figure 2D), and had better isovolumic systolic and diastolic function (Figure 2E) compared with identically treated wild-type C57 controls. Together, these studies show that calpain activation in ischemic myocardium contributes to adverse outcomes and raised the possibility that calpain cleavage of PKCα plays a contributory role.

Calpain 1 Cleaves PKCα at Multiple Sites Within the V3 Hinge Region

In mouse hearts, PKCα impairs β-adrenergic receptor responsiveness, depresses contractility, and predisposes to heart failure.4,5 In this context, we considered that there were 2 possible consequences of calpain-mediated PKCα fragmentation. First, calpains might “rescue” the PKCα phenotype by degrading and inactivating PKCα.12–14,29 Alternatively, calpain proteolysis might produce novel biological effects through independent activity of the fragments.29 To evaluate the latter possibility, it was necessary to define the daughter fragments by identifying the calpain cleavage site(s). Nishizuka and colleagues group reported putative calpain cleavage sites between amino acids K316 and V317 and between R324 and K325.26 Therefore, we initially mutated human PKCα V317 and K325 to prolines but found no effect on proteolysis by calpain 1 (Figure 3A). We next substituted alanines for K316 and V317 or R324 and K325 (in pairs), but these mutations also did not eliminate calpain 1–mediated PKCα cleavage (Figure 3A). Likewise, simultaneous mutation of all 4 amino acids failed to prevent calpain cleavage (Figure 3A). These results suggest that the predictions of Nishizuka and colleagues are overly restrictive.

Another view is that calpains recognize any of several undecapeptides in their targets and cleave at variable positions relative to these domains.30 Accordingly, we performed alanine substitution mutagenesis of the 2 halves of the PKCα V3 hinge region, en bloc, and also generated a mutant that included both alanine cassettes (double cassette). Purified calpain-1 was able to cleave both the PKCα cassette 1 and cassette 2 mutants, but not the double cassette mutant (Figure 3B). Together, these studies reveal promiscuity of calpain 1 proteolysis within the PKCα V3 hinge domain but exclude calpain 1 cleavage sites outside of the V3 region of PKCα. Thus, any calpain 1–mediated PKCα cleavage events will separate intact N-terminal regulatory and C-terminal catalytic kinase fragments.

PKCα N- and C-Terminal Fragments Translocate to Different Subcellular Domains

We examined the subcellular distribution of PKCα peptides corresponding to the calpain cleavage fragments after transient transfection in HEK293 fibroblasts. Full-length PKCα was primarily cytosolic in serum-deprived cells but translocated to cell membranes after treatment with 100 nmol/L phorbol myristate acetate (PMA) (Figure 4A). The N-terminal PKCα peptide (PKCα-NT) appeared in a punctate distribution primarily in the cytosol and showed no translocation after PMA, whereas the C-terminal peptide (PKCα-CT) showed a groundglass cytosolic appearance in unstimulated cells and translocated to cell membranes after PMA (Figure 4A).

The N-terminal and C-terminal domains of PKCs are tethered via intramolecular hydrogen bonds.10 We tested whether the free N- and C-terminal peptides interacted, which might have functional implications, by cotransfecting them in HEK293 cells, labeling them with different fluorophores, and performing confocal microscopy at baseline and after PMA treatment. Although PKCα-NT and -CT were cytosolic in unstimulated cells, they did not colocalize (Figure 4B, merged image). After PMA treatment, the 2 peptides were distributed in...
different subcellular compartments (Figure 4B). Thus, calpain-mediated PKC/H9251 proteolysis generates 2 free peptides exhibiting different subcellular compartmentation and translocation.

PKCα Fragments Are Stable in Myocardium and Have Functional Consequences

Having determined that calpain cleavage of PKCα separates the protein into 2 approximately equal-sized N-terminal and C-terminal halves, we next considered that any biological function of the fragments depended first on whether they were retained in cardiac myocytes. As this is an area of some controversy, we directly addressed the issue by conditionally expressing the 2 PKCα calpain fragmentation products and their parent, full-length PKCα, in mouse hearts.

Because a cardiac-specific (nonconditional) PKCα transgenic mouse was previously described, and has a phenotype similar to our conditional PKCα transgenic (see below), we propagated and fully phenotyped a single conditional full-length PKCα mouse line. Three independent lines of conditional PKCα N-terminal fragment (PKCαNT) and four lines of conditional PKCα C-terminal fragment (PKCαCT) mice were crossed to the tet-regulator transgene line, as required for protein expression. Of these, 2 lines of each were selected for phenotypic analysis based on initial studies showing levels of transgenic protein expression that were roughly equivalent to those of our full-length PKCα mouse (Online Figure I and Online Table I, available in the Online Data Supplement at http://circres.ahajournals.org). Full-length PKCα expressed at 8-fold endogenous levels (Figure 5A) produced mice with dilated (Figure 5B) and hypokontrictile (Figure 5C and 5E) hearts, having greatly diminished β-adrenergic responsiveness (Figure 5D). This phenotype is

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**Figure 2. Myocardial calpain 1 exacerbates ischemic cardiac injury.**

A, Infarct size 24 hours after left anterior descending artery occlusion in calpain 1 transgenic (n=6) and control (n=9) mice. Inset is calpain 1 immunoblot. Probability value is by Student’s t test. B, Twenty-four-hour survival after myocardial infarction in calpain 1 transgenic mice (n=20) and controls (n=19). Probability value is by log rank test. C, Left, TUNEL-stained left ventricular sections (control, left; calpain 1 transgenic, right) 24 hours after surgical coronary artery occlusion. Original magnification, ×1. Right, Quantitative analysis of TUNEL positivity in the remote, noninfarcted myocardium 24 hours after coronary occlusion; non indicates noninfarcted controls. Probability value is Tukey’s post hoc test after 1-way ANOVA. All values are means ± SEM. D, Representative infarcts of C57 control (wild-type [WT]) and calpain 1 knockout (KO) mice. E, Quantitative infarct size and functional data. White bars indicate WT; black bars knockout (n=9 per group for infarct and basal function studies, and n=5 per group for after dobutamine; probability values by Student’s t test).
similar to that previously described for both intrinsic cardiac PKCα activation and ~5-fold constitutive MYH6-driven cardiac PKCα overexpression.

Expression of PKCαNT at levels similar to full-length PKCα (Figure 5A) had no impact on cardiac size (Figure 5C) or ejection performance (Figure 5C and 5E), although adrenergic responsiveness was slightly diminished (Figure 5D). Remarkably, and despite expression levels lower than those for full-length PKCα or PKCαNT (Figure 5A), PKCαCT induced massive biventricular dilation (Figure 5B and Figure 5E, right) and severely depressed ejection performance (Figure 5C). Furthermore, the characteristic β-adrenergic unresponsiveness seen with cardiomyocyte-autonomous PKCα activation (Figure 5D and elsewhere) was fully recapitulated in PKCαCT transgenic mice (Figure 5D). These results demonstrate that both the N-terminal and C-terminal peptides corresponding to PKCα calpain proteolytic fragments are retained in cardiac myocytes and that the C-terminal fragment of PKCα (but not the N-terminal fragment) produces a cardiac phenotype similar to, but more severe than, parent PKCα.

The C-Terminal Fragment of PKCα Is a Dysregulated Myocardial Serine/Threonine Kinase

In vitro studies have suggested that C-terminal calpain cleavage fragments of some PKCs can act as unregulated and constitutively active kinases. To determine whether the PKCα C-terminal peptide exhibited kinase activity, we used DiGE and ProQ phosphoprotein staining. Nontransgenic mouse myocardium exhibits a characteristic phosphoprotein pattern (Figure 6, red). PKCαNT myocardium showed a similar phosphoprotein pattern (see below and data not shown). In contrast, PKCα transgenic myocardium exhibited a number of newly phosphorylated proteins (Figure 6, green), virtually all of which were also observed in PKCαCT myocardium (Figure 6, blue). PKCαCT myocardium was similar to PKCα but additionally revealed phosphopeptides that were increased in staining intensity from, or not observed at all in, full-length PKCα hearts (blue spots in Figure 6, merged).

These data suggested that cardiomyocyte-expressed PKCα CT possesses kinase activity that mimics that of its parent PKCα but may also be partly dysregulated and/or mistargeted. To identify differentially phosphorylated proteins, we selected several spots of interest and identified them by mass spectroscopy (Online Table II). Two phosphoprotein spots that were more prominent in PKCα transgenic than control hearts (arrowheads, Figure 6) were identified as heat shock protein 90 (HSP90β) and phosphoglucomutase, which are serine/threonine-phosphorylated in striated muscle. MyBP-C, α cardiac actin, and the regulatory subunit of protein phosphatase 2a (PP2A pR65) were each phosphorylated by both full-length and the C-terminal fragment of PKCα, whereas α-actinin, annexin II, and calsarcin 1 (myozenin-1) appeared to be phosphorylated only in PKCαCT hearts.

PKCs are serine/threonine kinases, and the ProQ phosphoprotein staining procedure we used to map and quantify protein phosphorylation by 2D gel electrophoresis does not distinguish between serine/threonine phosphorylation and tyrosine phosphorylation. Therefore, we also performed 2D immunoblotting using an anti-phosphoserine/threonine antibody. The protein pattern was consistent between myocardial samples (Figure 7A,
green), but differences in anti-phosphoserine/threonine immuno-reactivity were revealing. Compared with nontransgenic controls, PKCα/H9251 NT serine phosphorylation was similar. In contrast, serine/threonine phosphorylation was generally increased in PKCα/H9251 and PKCα/H9251 CT myocardium (Figure 7, red), reproducing the findings from phosphoprotein staining. Thus, observed differences in protein phosphorylation in PKCα/H9251 and PKCα/H9251 CT hearts compared with control and PKCα/H9251 NT hearts are attributable to increased serine/threonine phosphorylation.

The C-Terminal Fragment of PKCα Abnormally Phosphorylates MyBP-C

Collectively, the above data indicate that cardiac ischemia can activate PKCα-like activity by calpain-mediated formation of the free PKCα C-terminal fragment. The exaggerated phenotype and phosphoprotein profile of PKCαCT hearts further suggested that PKCαCT might have effects beyond those of activity-constrained intact PKCα. We pursued evidence to support this idea by comparing phosphorylation of myosin binding protein C (which mass spectroscopy had identified as differentially phosphorylated; see Figure 6 and Online Table II) in PKCα and PKCαCT hearts. MyBP-C is a polyphosphorylated substrate of at least 3 kinases, PKC, calmodulin kinase II, and PKA,34 and increased MyBP-C phosphorylation contributes to postischemic myocardial dysfunction.35

We first confirmed the mass spectroscopic identity and differential phosphorylation of MyBP-C by 2D immunoblotting (Figure 8A). Next, we repeated the DiGE studies using a narrower pH gradient for better protein separation. MyBP-C migrated as 9 spots on the 2D gels (Figure 8B), and the protein masses were identified by mass spectroscopy. Arrowheads indicate representative uniquely or differentially phosphorylated proteins on the PKCα and PKCαCT blots. Proteins phosphorylated by PKCα, but not in control, are green in control/PKCα merge (bottom left). Proteins phosphorylated by PKCαCT, but not by either control or PKCα, are blue in control/PKCα/PKCαCT merge (bottom right). Protein mass spectroscopic identities are indicated on triple merged image.
A profile was shifted toward a lower pH in PKCoCT hearts (Figure 8B). ProQ staining confirmed that this shift reflected increased MyBP-C phosphorylation (Figure 8B, bottom). Phosphorylation mapping of canine MyPBC3 has indicated previously that the 2 most basic spots are nonphosphorylated MyBP-C, spots 3 to 5 are MyBP-C–phosphorylated at S282, and spots 6 to 9 represent various combinations of polyphosphorylated MyBP-C at S273, S282, S284, S302, and S307 (mouse amino acid numbering). Analysis of individual spots also suggested that the polyphosphorylated MyBP-C species were increased in PKCoCT hearts (Figure 8C), and this was confirmed by phospho–MyBP-C–specific immunoblotting (Figure 8D). Thus, PKCoCT phosphorylation of MyBP-C was greater than that of intact PKCo, suggesting promiscuous activity of the free catalytic fragment.

**Discussion**

PKC family members are activated as part of many neurohormone receptor signaling pathways linked to cardiac disease. Here, we report that limited calpain 1–mediated proteolytic cleavage of PKCo during ischemia generates a catalytically active kinase fragment, independent of conventional receptor pathways. PKCo proteolysis was detected only after ischemia/reperfusion in either normal or calpain 1 transgenic hearts, demonstrating a requirement for ischemia that presumably activates calpain via increased free calcium. Through conditional overexpression of the predicted calpain PKCo proteolysis products, we showed that both the N- and C-terminal PKCo calpain fragments are retained in the myocardium. The N-terminal fragment exhibited no kinase activity, had no effect on baseline cardiac structure and contractile performance, and had only a modest negative impact on β-adrenergic responsiveness. In contrast, the C-terminal PKCo fragment exhibited broad kinase activity and was a potent negative regulator of myocardial function, independent of ischemia or calpain activation. The extent of myocardial protein phosphorylation and the deleterious cardiac consequences of the free PKCo catalytic fragment were all greater than those of PKCo itself.

The present findings invert the accepted paradigm for PKC activation/inactivation. Classically, agonist stimulation of Gq-coupled neurohormone receptors activates phospholipase C that promotes the hydrolysis of phosphatidylinositol bisphosphate to sn-1,2-diacylglycerol (DAG) and inositol(1,4,5)trisphosphate (IP3). The latter activates IP3 receptors to stimulate release of calcium from endoplasmic and sarcoplasmic reticulum. Calcium and DAG bind to specific sites in the N-terminal regulatory domain of conventional PKCs, altering their structures and weakening intramolecular bonds that hold the N- and C-terminal halves together like 2 halves of a clam shell. Consequently, the PKC protein unfolds at the intervening hinge domain. Physical separation of N-terminal regulatory domains from their C-terminal substrate-binding and membrane-targeting (RACK-binding) domains disinhibits the kinase, permitting membrane translocation and substrate phosphorylation. This process not only activates PKC but also begins the process for its inactivation through proteolytic degradation. Indeed, before the development of specific pharmacological inhibitors, PKC downregulation by prolonged exposure to phorbol ester DAG mimetics was commonly used for in vitro studies. Notwithstanding that calpains are widely known as effectors of PKC inactivation
through degradation,\textsuperscript{12,37} this is opposite to the effect that we observed. Whereas parental myocardial PKC\textalpha\ can certainly be removed by the proteolytic actions of calpain, we found that calpain 1 produced a catalytically active daughter PKC\textalpha\ fragment corresponding to the PKM\textalpha\ of Nishizuka and colleagues.\textsuperscript{26} Thus, the mechanism for PKC “inactivation by proteolytic degradation” actually increases pathological signaling by producing a kinase that is not subject to normal physiological constraints.

PKC\textalpha\ is upregulated \textasciitilde80\% in human and experimental cardiac hypertrophy, which contributes to impaired \textbeta\-adrenergic signaling.\textsuperscript{38,39} Increased expression or activity of myocardial PKC\textalpha\ produces contractile dysfunction and cardiomyopathy phenotypes,\textsuperscript{4,5} whereas its genetic ablation or pharmacological inhibition enhances contractility and protects against cardiomyopathy.\textsuperscript{5–7} Although PKC-mediated phosphorylation of titin may regulate myocardial stiffness,\textsuperscript{40} and it may exert an indirect effect of on phospholamban/SERCA function through phosphorylation of protein phosphatase inhibitor 1,\textsuperscript{5} the myocardial substrates of PKC\textalpha\ are largely unknown and the mechanisms by which it causes cardiomyopathy are poorly understood. The present studies begin the process of identifying in vivo cardiac PKC\textalpha\ substrates and further indicate that the PKC\textalpha\ C-terminal fragment has a broader kinase substrate profile than the parent enzyme.

All of the phosphoproteins we identified (or their skeletal muscle counterparts) have previously been detected in the human muscle phosphoproteome and are known to be phosphorylated on one or more serine/threonine residues.\textsuperscript{33} Our studies showed that full-length PKC\textalpha\ and the free PKC\textalpha\ C-terminal fragment phosphorylated many of the same cardiac substrates; the chaperone HSP90\textbeta\ is an example. However, we identified 2 catalytic effects that functionally distinguish the C-terminal fragment from full-length PKC\textalpha.\textsuperscript{5} The first is hyperphosphorylation of normal PKC\textalpha\ substrates. This was specifically demonstrated for the myosin binding protein MyBP-C, which showed a stepwise increase in phosphorylation from control hearts, to those expressing PKC\textalpha, to those expressing PKC\textalpha\CT. It is of special interest that a MyBP-C hyperphosphorylation pattern similar to ours is seen after myocardial ischemia and is prevented by ischemic preconditioning.\textsuperscript{35} MyBP-C phosphorylation by PKC\textalpha\ was also recently proposed as contributing to dilated cardiomyopathy.\textsuperscript{41} Thus, MyBP-C is one potential mediator of cardiomyopathic effects of PKC\textalpha\CT.

The second distinct effect of PKC\textalpha\CT was phosphorylation of proteins that were not usual substrates for full-length PKC\textalpha, such as the cytoskeletal protein \textalpha\-actinin. Indeed, PKC does not normally phosphorylate \textalpha\-actinin,\textsuperscript{42} although PKN, a Rho-activated serine/threonine kinase having a catalytic domain similar to the PKCs (and therefore to the PKC\textalpha\ C-terminal fragment), does.\textsuperscript{33} Excessive phosphorylation of normal substrates and phosphorylation of atypical substrates are the expected consequences of a catalytic kinase lacking normal regulatory and targeting domains.

Calpain-mediated proteolytic cleavage of PKCs into PKMs was described years ago,\textsuperscript{26} but the physiological relevance of these events for in vivo cell signaling has been discounted because of the presumed instability of the proteolytic frag-
ments.33 Indeed, PKM produced via the actions of calpain 2 is highly unstable, whereas that produced by calpain 1 can persist even after complete PKC degradation.27 Recent studies of PKM production in PKC-rich neural tissue have stimulated interest in the possibility that calpain cleavage regulates PKC signaling in learning and memory.45 The mollusk Aplysia exhibits calpain-dependent proteolysis of PKC and generation of persistently active PKM that is essential for intermediate memory.46 In these studies, pharmacological PKC inhibitors that targeted the catalytic domain of PKC prevented the PKC effect (intermediate-term memory), whereas the PKC inhibitor, calphostin C, which interacts with the regulatory domain, was ineffective. Because intermediate-term memory is a sustained response, these findings suggest special importance for generation of the constitutively active catalytic PKM fragment for prolonged signaling. Similar findings were recently described in rat brain, where calcium influx through voltage-dependent calcium channels generated PKM that produced agonist-independent burst firing of dopamine cells.47 Again, this response was inhibited by chelerythrine, which acts at the PKC catalytic domain, but not by calphostin C, which acts on the DAG binding site of the regulatory domain.

The intersection of PKC and calpain activation in calcium-overloaded ischemic tissue represents the convergence of 2 potent signaling pathways. Myocardium may be especially sensitive to PKC–calpain functional interactions, because the heart is adversely impacted by PKCα signaling and contains abundant calpain 1 that is activated during ischemia.16 The intent of our studies was to determine whether calpain-mediated processing of PKCα occurs in ischemic hearts, to ascertain how this affected myocardial kinase activity, and to assess the possible consequences of this novel signaling event on the heart. Thus, the extent to which calpain-mediated PKCα cleavage contributes to postischemic myocardial dysfunction and progression to heart failure is unknown at this time. However, because PKCα is the most abundant PKC isoform in myocardium,4 calpain 1 is activated and upregulated following myocardial ischemia.48–50 and the PKCα C-terminal peptide can clearly be retained for significant periods in myocardium, it is likely that calpain-generated PKMα is detrimental to the postischemic heart.

The present observations may have relevance to ongoing evaluations of therapeutics designed to inhibit cardiac PKCα signaling. Catalytically active PKMα generated by calpains activated during ischemia/reperfusion will not be sensitive to PKCα inhibitors, such as calphostin C, that target regulatory domains.46,47 Our understanding is that PKCα inhibitors currently being evaluated in heart failure, such as Ro-32-0432 and RO–31-8220, act at the catalytic site. Their reported benefits may therefore accrue, in part, from PKMα inhibition.6

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**Disclosures**
None.

**References**


Novelty and Significance

What Is Known?

- Protein kinase (PKC) signaling is activated by neurohormonal G protein–coupled receptor pathways stimulated during cardiac stress and is detrimental to heart function.
- Calpain cysteine proteases are activated by calcium released from the myocardium damaged by ischemia, and they injure the heart by actively degrading myocardial proteins.

What New Information Does This Article Contribute?

- We have identified and characterized the crosstalk between the calpain and PKC stress response pathways, providing for receptor–independent activation of cardiac PKC signaling.
- Cleavage of PKCα by calpain 1, specifically in ischemic hearts, produces a catalytically active kinase fragment that, because of the proteolytic removal of its normal regulatory domains, is not subject to usual physiological constraints.
- The unregulated catalytic fragment exhibits promiscuous kinase activity and exerts detrimental effects on heart structure and function greater than those of its parent, PKCα.

Calpains and PKC are both calcium-activated stress signaling pathways that independently affect cardiac function; however, the implications of their potential interaction had not been explored. Calpains typically degrade substrate proteins. We identified multiple adjacent calpain cleavage sites in PKCα that, rather than degrading the kinase, liberate a catalytically active fragment that is unregulated and mistracted, exhibiting promiscuity of substrate phosphorylation. These findings describe a receptor-independent mechanism that activates a potent PKC-derived rogue kinase. The calpain-generated PKCα catalytic fragment is only observed after ischemic injury, is stable in the myocardium, induces hyperphosphorylation of normal PKCα substrates and misdirected phosphorylation of nonsubstrate proteins, and produces aggressive cardiomyopathy. Our results have implications for cardiac therapeutics. They suggest that inhibiting PKC signaling at the level of neurohormone receptors or calcium activation is unlikely to affect the calpain–PKC pathway; instead, direct suppression of PKC catalytic activity might be a more effective therapeutic approach.
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