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# Revisited and Revised: Is RhoA Always a Villain in Cardiac Pathophysiology?

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**Abstract** The neonatal rat ventricular myocyte model of hypertrophy has provided tremendous insight with regard to signaling pathways regulating cardiac growth and gene expression. Many mediators thus discovered have been successfully extrapolated to the *in vivo* setting, as assessed using genetically engineered mice and physiological interventions. Studies in neonatal rat ventricular myocytes demonstrated a role for the small G-protein RhoA and its downstream effector kinase, Rho-associated coiled-coil containing protein kinase (ROCK), in agonist-mediated hypertrophy. Transgenic expression of RhoA in the heart does not phenocopy this response, however, nor does genetic deletion of ROCK prevent hypertrophy. Pharmacologic inhibition of ROCK has effects most consistent with roles for RhoA signaling in the development of heart failure or responses to ischemic damage. Whether signals elicited downstream of RhoA promote cell death or survival and are deleterious or salutary is, however, context and cell-type dependent. The concepts discussed above are reviewed, and the hypothesis that RhoA might protect cardiomyocytes from ischemia and other insults is presented. Novel RhoA targets including phospholipid regulated and regulating enzymes (Akt, PI kinases, phospholipase C, protein kinases C and D) and serum response element-mediated transcriptional responses are considered as possible pathways through which RhoA could affect cardiomyocyte survival.

**Keywords** RhoA · Cardiomyocyte Ischemia · ROCK

## G $\alpha_q$ and RhoA Signaling Pathways in Cardiac Hypertrophy

There is considerable evidence that G-protein coupled receptors (GPCRs) that interact with the heterotrimeric G-protein, G $\alpha_q$ , mediate cardiac hypertrophy (see Fig. 1). In neonatal rat ventricular myocytes (NRVMs), GPCR agonists such as norepinephrine (NE), phenylephrine (PE), and endothelin 1 (ET-1), acting through  $\alpha_1$ -adrenergic and ET-1 receptors coupled to G $\alpha_q$ , induce cardiac hypertrophy as evidenced by fetal gene expression, myofilament organization, increased protein synthesis, and cardiomyocyte enlargement [1–7]. *In vivo* studies subsequently demonstrated that transgenic expression of G $\alpha_q$  induces hypertrophy, as does G $\alpha_q$  expression in NRVMs [8–10]. Development of hypertrophy in mice subjected to transverse aortic constriction (TAC) was subsequently shown to be prevented by transgenic expression of a peptide inhibitor that blocks GPCR coupling to G $\alpha_q$  [11], or by genetic deletion of the alpha ( $\alpha$ ) subunit of G $\alpha_q$  and its homolog G $\alpha_{11}$  [12]. The best known effector of G $\alpha_q$  is phospholipase C (PLC) [13, 14], and accordingly, signals generated through phosphoinositide hydrolysis, including activation of protein kinase C (PKC) and of Ca<sup>2+</sup>-regulated enzymes such as calcineurin and CaMKII have been considered to serve as downstream mediators of GPCR effects on hypertrophic gene expression and cell enlargement [15, 16].

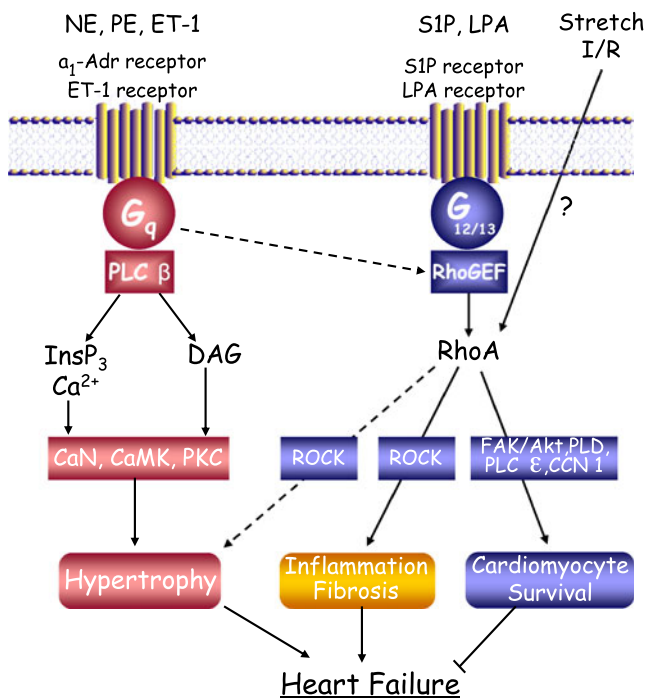
Studies carried out *in vitro* and subsequently *in vivo* also suggested that a low-molecular-weight G-protein, RhoA, plays a role in development of cardiac hypertrophy. Early studies from our group and others showed that in the NRVMs model of hypertrophy, agonist-induced increases

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**Fig. 1** GPCRs signaling in hypertrophy, survival, and failure. *Dashed lines* indicate pathways that the authors do not consider to be predominant. *Abbreviations:* NE norepinephrine, PE phenylephrine, ET-1 endothelin-1, S1P sphingosine 1-phosphate, LPA lysophosphatidic acid, I/R ischemia/reperfusion, InsP<sub>3</sub> inositol-1,4,5-triphosphate, DAG diacylglycerol, PLC phospholipase C, RhoGEF rho guanine nucleotide exchange factor, PKC, protein kinase C, CaMK, calcium-calmodulin-dependent kinase, CaN, calcineurin, ROCK rho-associated coiled-coil containing protein kinase, FAK focal adhesion kinase, PLD phospholipase D

in cell size, protein expression, and actin organization (all hallmarks of hypertrophy) could be attenuated by treatment with the C3 exoenzyme, which ribosylates and inhibits RhoA function [17, 18], or by expression of a dominant-negative form of RhoA [19]. Hypertrophic effects of RhoA were demonstrated to be transduced through activation of Rho-associated coiled-coil containing protein kinase (ROCK), a well-characterized RhoA effector [20–24]. RhoA and ROCK have also been demonstrated to be transducers of hypertrophy induced by static or pulsatile stretch of NRVMs [25, 26]. Our laboratory showed involvement of RhoA in MAP kinase translocation to the nucleus and in cardiomyocyte enlargement induced by stretch [25], while others have demonstrated that stretch-induced regulation of hypertrophy-associated gene expression is abolished following transfection with RhoA antisense oligonucleotides [26].

RhoA and ROCK have also been implicated in hypertrophy induced by pressure overload (TAC) or in vivo agonist infusion. There is rapid activation of RhoA and ROCK in adult rat hearts subjected to pressure overload [27]. Moreover, recent work using a similar pressure

overload model showed that ROCK inhibition reduced the hypertrophic response and collagen deposition (a result of fibrosis), as well as improving cardiac function [28]. Treatment with the ROCK inhibitor fasudil (HA-1077) also blunted the hypertrophic response to angiotensin II (Ang II) infusion in rats, a treatment associated with ROCK activation as assessed by phosphorylation of ezrin/radixin/moesin (ERM) proteins [29]. These findings support the involvement of RhoA/ROCK signaling in development of hypertrophy in vivo.

The relative importance of, and relationship between, G $\alpha_q$  and RhoA signaling pathways in agonist and TAC-induced hypertrophy has not been extensively analyzed. Is RhoA a downstream target of G $\alpha_q$  signaling or does RhoA initiate a distinct and parallel hypertrophic signaling pathway? We originally proposed that RhoA could be activated downstream of G $\alpha_q$  in NRVM hypertrophic pathways [19], although we had no specific mechanistic insights into how this would occur. RhoA is activated by guanine nucleotide exchange factors (GEFs), proteins that catalyze exchange of guanosine 5c-diphosphate (GDP) for guanosine 5c-triphosphate (GTP) on RhoA [30]. The GTP-bound RhoA is the active form that interacts with and regulates effectors such as ROCK to elicit downstream responses [31, 32]. While it has been clear for many years that certain GPCR agonists can cause RhoA activation, the GEFs acting downstream of GPCRs have only recently been identified. Among these are GEFs such as the p63 rho GEF (RhoGEF), shown to bind and be regulated by G $\alpha_q$  [33–35]. Discovery of G $\alpha_q$ -regulated GEFs provides a means by which GPCRs that stimulate G $\alpha_q$  could also lead to RhoA activation and RhoA-mediated hypertrophy. Perhaps the newly discovered protective effects of cardiac  $\alpha_1$  adrenergic receptors (Simpson, unpublished observations) reflect activation of a RhoA signaling pathway.

Notably, however, the best described hypertrophic agonists (NE, PE, and ET-1) are not nearly as efficacious at activating RhoA as are another set of ligands, including sphingosine 1-phosphate (S1P), lysophosphatidic acid (LPA), thrombin, and thromboxane A<sub>2</sub>. The receptors for this latter group of ligands couple not only to G $\alpha_q$  but also with high efficiency to the newest member of the heterotrimeric G-protein family, G $\alpha_{12}$  and its family member G $\alpha_{13}$  [36, 37]. Indeed, initial insights into how GPCRs activate RhoA emerged from seminal papers demonstrating that a particular GEF, the p115RhoGEF, interacts directly with G $\alpha_{12}$  and G $\alpha_{13}$  [38, 39]. It is now clear that the interaction of G $\alpha_{12}$  or G $\alpha_{13}$  with other RhoGEFs including leukemia-associated RhoGEF(LARG) and PDZ-RhoGEF leads to their activation [30, 40, 41]. There is also an A kinase-anchoring protein (AKAP-Lbc) that contains a RhoGEF domain and mediates RhoA activation in cardiomyocytes in response to agonists such as LPA and PE,

[42, 43]. It is now well accepted that much as the effector for  $G\alpha_s$ -coupled receptors is adenylyl cyclase and that for  $G\alpha_q$ -coupled receptors is PLC $\beta$ , RhoGEFs and RhoA activation serve as the primary effector for signaling by GPCRs that couple to  $G\alpha_{12/13}$  proteins. Ligands such as S1P and LPA, by activating GPCRs that couple to  $G\alpha_{12}$  or  $G\alpha_{13}$  and hence to RhoGEFs, are very effective initiators of RhoA signaling pathways.

Interestingly, recent studies challenge the hypothesized role of  $G\alpha_{12/13}$ , RhoA, and ROCK in development of hypertrophy. Expression of  $G\alpha_{12}$  in NRVMs has been shown to induce a hypertrophic response [18, 44, 45]. Moreover a recent study of transgenic mice engineered to express an inhibitor of  $G\alpha_{12/13}$  (RGS domain of p115GEF) in a cardiac-specific manner did not show decreases in hypertrophy in response to pressure overload [46]. Genetic deletion of  $G\alpha_{12/13}$  also fails to block hypertrophy in vivo (S. Offermanns, personal communication). These findings contrast with those of comparable experiments cited above in which TAC-induced hypertrophy was inhibited when  $G\alpha_q$  signaling was prevented [12, 47]. Recent studies also show no inhibition of pressure overload-induced hypertrophy in mice in which ROCK1, the RhoA target suggested to mediate hypertrophy in NRVMs, is genetically deleted [48, 49]. Several lines of evidence from our laboratory also argue that RhoA signaling does not lead to cardiac hypertrophy. For example, we observe no difference in hypertrophy induced by pressure overload in mice in which S1P<sub>2</sub> or S1P<sub>3</sub> receptors are genetically deleted, although we know that stimulation of S1P receptors leads to robust RhoA activation in cardiomyocytes [50, 51] and that RhoA activation occurs through S1P<sub>2</sub> and/or S1P<sub>3</sub> receptors [52]. We have also observed that cardiac-specific inducible RhoA expression does not lead to hypertrophy in mice followed for up to 1 year (Xiang et. al, manuscript in preparation). Thus, reevaluation with new models indicates that RhoA signaling is neither sufficient for the induction of cardiac hypertrophy nor necessary for that induced by pressure overload in vivo. If RhoA is not a critical player in development of in vivo hypertrophy, is there an alternative physiological role for RhoA activation and the agonists/interventions that induce RhoA activation in the heart?

## Heart Failure

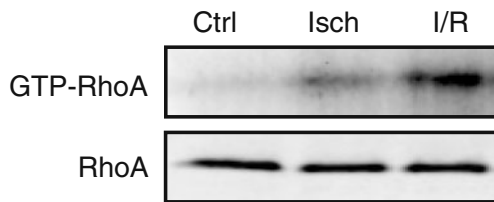
Cardiac hypertrophic responses can become maladaptive if the initial cardiac insult persists. The mechanisms responsible for the transition from compensatory to maladaptive hypertrophy and remodeling are not well understood, although various molecular mechanisms have been suggested to underlie this transition. A role for RhoA activation in the transition from hypertrophy to dilation

and heart failure is suggested by several in vivo findings. One is that a lethal dilated cardiomyopathy develops in cardiac-specific RhoA transgenic mice [53]. More recent studies using a tyrosine phosphatase knockout mouse also showed RhoA-mediated cardiac dilation, suggesting a role for RhoA in the development of cardiomyopathy [54]. Genetically altered mouse models have also implicated ROCK in the development of heart failure. Thus, whereas ROCK1 null and heterozygous null mice show no difference in development of hypertrophy following pressure overload or Ang II infusion, they have significantly less fibrosis and reduced expression of a variety of extracellular matrix (ECM) proteins and fibrogenic cytokines [48, 55]. Similarly, there is improved cardiac function in the  $G\alpha_q$  transgenic model of dilated cardiomyopathy when these mice are crossed with mice in which ROCK1 is deleted [49]. Taken together these studies implicate RhoA/ROCK signaling in the transition from compensatory hypertrophy to heart failure.

Both expression and activity of RhoA and ROCK have been noted to increase in a variety of cardiovascular disease models, including myocardial infarction and pressure overload [27, 56–58]. ROCK1 is activated not only by RhoA binding but also through its cleavage, which is increased in human heart failure patients [59]. A maladaptive role of RhoA/ROCK signaling in the cardiovascular system in vivo is supported by several studies demonstrating that inhibitors of RhoA/ROCK diminish diastolic contractile dysfunction induced by pressure-overload or reperfusion injury [28, 60, 61]. Key to interpreting these findings, however, is that the sites for maladaptive ROCK signaling are not clearly defined. In studies using pharmacologic inhibitors of ROCK, as well as in conventional knockout mouse models, ROCK function would be inhibited not only in cardiomyocytes but also in fibroblasts, endothelial, and inflammatory cells. RhoA/ROCK signaling pathways are well-established mediators of changes in migration, proliferation, and gene expression in these cell types [62–66]. Accordingly, RhoA- and ROCK-mediated responses in noncardiomyocytes likely contribute to the detrimental effects of RhoA signaling in cardiovascular disease.

## Ischemic Injury

Ischemia/reperfusion (I/R) damage occurs when interrupted blood flow is followed by restored circulation, resulting in oxidative stress, mitochondrial dysfunction, inflammation, and tissue damage. I/R also activates numerous intracellular signaling pathways, some deleterious, but others protective [67]. We have observed marked activation of RhoA in response to I/R in isolated perfused mouse hearts (Fig. 2).



**Fig. 2** RhoA is activated by ischemia/reperfusion in the perfused mouse heart. Isolated adult mouse hearts were retrograde perfused using the Langendorff method. Hearts were subjected to continuous perfusion (Ctrl), ischemia (Isch) for 30 min, or ischemia for 30 min and reperfusion for 60 min (I/R). Hearts were frozen, homogenized, and assessed for total RhoA in the lysate and activated RhoA based on pull-down with GST-rhotekin

Whether this occurs through activation of a RhoGEF in response to released mediators or as a direct result of oxidative stress is not known. Interestingly, a recent paper provided evidence that RhoA can be directly activated by reactive oxygen species, via a mechanism involving critical cysteine residues present in a redox-sensitive motif [68]. Published studies using an *in vivo* rat I/R model demonstrate increased expression of RhoA and activity of ROCK following 30 min of coronary occlusion followed by 24 h of reperfusion [60]. In this model, infarct size was reduced by inhibiting ROCK with Y-27632. A similar study carried out using an *in vivo* mouse I/R model also showed decreased infarct size and significantly less inflammation in mice treated with ROCK inhibitors compared with control, suggesting a deleterious role of RhoA/ROCK signaling in ischemic injury [61, 69].

What are the mechanisms by which ROCK inhibition could decrease I/R injury? A recent study showed that infarct size was not diminished by Y-27632 when wortmannin or nitro-L-arginine methyl ester were also present, suggesting that protective PI3K/Akt/NO signaling pathways are necessary [61]. Another study demonstrated that I/R decreased expression of the antiapoptotic Bcl-2 protein and that this did not occur in Y-27632-treated hearts [60]. Consistent with a role of ROCK in apoptosis, Y-27632-treated animals showed reduced TUNEL-positive nuclei in the infarcted regions [61]. Inflammatory responses induced by I/R are also abrogated by inhibition of ROCK with Y-27632 suggesting that RhoA/ROCK effects on inflammatory gene expression contribute to cardiovascular injury after I/R [61, 69]. Finally ROCK inhibition with Y-27632 or fasudil (HA-1077) was shown to decrease fibrosis following myocardial infarction in both mouse and rat models [56, 58], indicating that ROCK contributes either directly or indirectly to proliferation of cardiac fibroblasts in ischemic disease. Thus, there are numerous sites and mechanisms through which Rho/ROCK signaling could be deleterious and account for the salutary effect of ROCK inhibitors on I/R injury and development of heart failure.

## RhoA Regulation of Cardiomyocyte Death and Survival

Cardiomyocyte loss by apoptosis and/or necrosis plays a crucial role in development of heart failure [70–72]. Our previous finding that cardiac-specific RhoA transgenic mice show spontaneous dilated cardiomyopathy [53] led us to hypothesize that cardiomyocyte cell death could be induced by sustained activity of RhoA. In subsequent work, we demonstrated that enhanced and sustained RhoA/ROCK signaling in NRVMs induces cardiomyocyte apoptosis [50]. Specifically, we demonstrated that expression of constitutively activated RhoA for 48–72 h activated a mitochondrial death pathway in association with a striking up-regulation, activation, and mitochondrial association of the proapoptotic Bcl family member, Bax [50].

Conversely, we found that more acute RhoA activation protected cardiomyocytes from apoptotic insult [51]. Expression of activated RhoA in NRVMs for less than 24 h did not induce apoptosis but rather protected cells against both peroxide and glucose deprivation-induced apoptosis. Protection was dependent on ROCK activity, cytoskeletal integrity, and the activation of focal adhesion kinase (FAK). FAK, which is known to be activated through integrin engagement with the ECM, has a number of distinct phosphorylation sites that enable binding to signaling molecules including Src, PI3K, and p130Cas [73, 74]. We demonstrated that the role of FAK as a protein scaffold is responsive to RhoA signaling in NRVMs, recruiting the p85 subunit of PI3K and activating the survival kinase Akt. Mechanical stretch, which has been shown to activate RhoA in cardiomyocytes, was also found to elicit FAK and Akt activation [26, 51]. Interestingly, cardiomyocyte-specific ablation of FAK increased infarct size and cardiomyocyte apoptosis in response to I/R [75], consistent with a role for FAK as a protective downstream target of RhoA signaling in cardiomyocytes (Fig. 3).

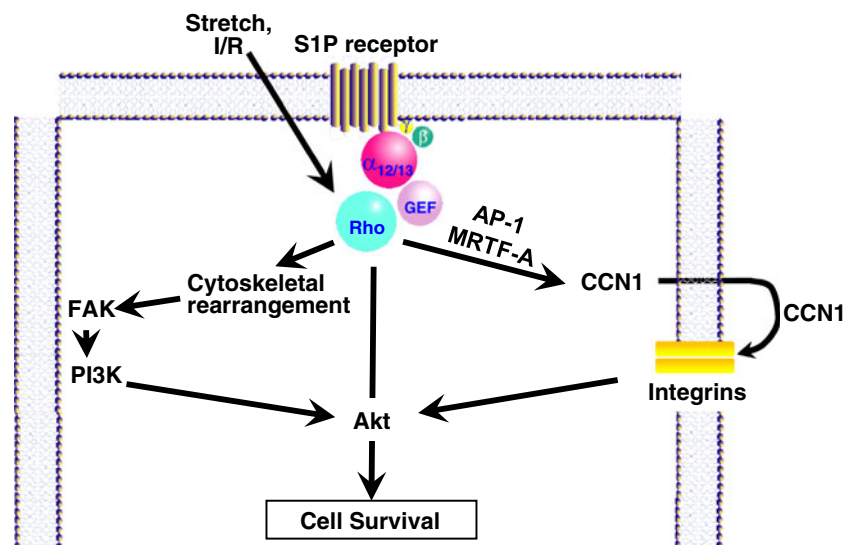
In summary, although there is much evidence that activation of ROCK is deleterious in the heart, RhoA may have the capacity to confer protection in cardiomyocytes by signaling through Akt or other effectors. Recent studies in cardiac and noncardiac cells have identified new targets through which activated RhoA can signal. These, as well as more established targets that have not been fully investigated, are described below as potential mediators of cardiomyocyte protection through RhoA signaling.

## RhoA and Phospholipid Signaling

### Phosphoinositide Synthesis

As described above, RhoA acts indirectly, through its well-known effects on cytoskeletal remodeling and FAK, to

**Fig. 3** RhoA-mediated protective signaling through Akt

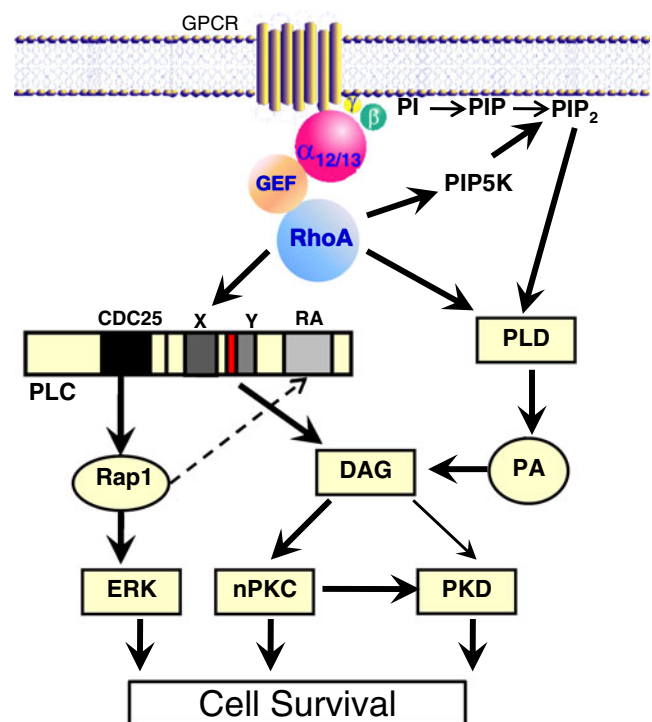


stimulate phosphoinositide signaling. There are, in addition, other phospholipid signaling pathways that are modulated through RhoA, several of which appear to be the direct result of RhoA interactions with phospholipid metabolizing enzymes (Fig. 4). One of the earliest effects described in mammalian cells was the regulation of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) synthesis via effects of RhoA on the synthetic enzyme PIP-5 kinase [76, 77]. RhoA-mediated changes in the synthesis and hence the level of PIP<sub>2</sub> can affect the ability of the cell to respond to integrins or GPCRs that signal via PLC-mediated PIP<sub>2</sub> hydrolysis [76, 78]. In addition to serving as a substrate for PLC, PIP<sub>2</sub> subserves myriad cellular functions including regulation of ion channels and cytoskeletal proteins and recruitment of signaling molecules to the cell membrane. Thus, one hypothesis is that effects of RhoA on PIP<sub>2</sub> levels can affect cell survival [79, 80].

#### PLC Activation

PLC epsilon (PLCε), the newest member of the PLC family, is uniquely positioned to serve as an integrator of signaling from GPCRs and small GTPases [81, 82]. This isoform of PLC is directly regulated by binding of the small GTPases Rap1 and RhoA [83–88]. There is, in contrast, no regulation by Gα<sub>q</sub>, the direct activator of the canonical PLC, PLCβ [89–91]. Accordingly PLCε is regulated by agonists that couple to Gα<sub>12/13</sub> and RhoA rather than those that couple to Gα<sub>q</sub> [83, 90]. Another critical feature of PLCε is that it contains an N-terminal CDC25 homology domain that functions as an exchange factor for small GTPases [85, 87, 92]. This allows the enzyme to function not only as a phospholipase (generating diacylglycerol [DAG] and inositol trisphosphate) but also as an activator of Rap1 (Fig. 4).

Two important functions downstream of Rap1 activation may be relevant to cardiomyocyte signaling. One is that active Rap1 could feedback on and thus continue to activate PLCε, contributing to sustained DAG production [83, 87, 93]. DAG plays an important role in activation of PKC and studies from the Smrcka laboratory demonstrate that the novel PKC isoform, PKCε, is in fact activated through PLCε in the heart [94]. Rap1 also activates ERK, and we have shown that PLCε contributes to sustained agonist-



**Fig. 4** RhoA-mediated protective signaling through phospholipid regulatory enzymes

induced ERK activation [83]. There is considerable evidence that ERK signaling is protective in many cell types including cardiomyocytes [95–97], as is PKC $\epsilon$  [98–102]. Thus, sustained activation of ERK or PKC $\epsilon$ , resulting from activation of PLC $\epsilon$ , could contribute to RhoA-mediated cardiomyocyte protection. Smrcka's laboratory also demonstrated that there is a significant level of PLC $\epsilon$  expression in the heart and that PLC $\epsilon$  is increased in human failing hearts and in animal models of pressure overload and isoproterenol-induced hypertrophy [103]. Their analysis of PLC $\epsilon$  knockout mice revealed decreased  $\beta$ -adrenergic receptor-induced contractility. In addition, they saw enhanced pathological hypertrophy and fibrosis in PLC $\epsilon$  knockout mice, leading to the suggestion that PLC $\epsilon$  protects against development of pathologic hypertrophy [103].

#### Phospholipase D Activation

While PLC activation is often considered to be the major mechanism for DAG generation, another phospholipase, phospholipase D (PLD) may be equally or more important. In contrast to PLC, PLD uses the more abundant phospholipid, phosphatidylcholine, as its substrate and initially produces phosphatidic acid (PA), which is then converted to DAG through the actions of lipid phosphatases (Fig. 4). Two mammalian PLD isozymes, PLD1 and PLD2, have been identified [33, 104–106]. There is abundant evidence that PLD1 (but not PLD2) is activated by the Rho family GTPases RhoA, Rac1, and Cdc42, with RhoA being the most efficacious [105, 107–109]. RhoA regulates PLD1 [108, 110–113] through direct interaction with its C-terminus [108, 110, 111, 114]. Activation of PLD1 could also occur indirectly through increased RhoA-mediated synthesis of PIP<sub>2</sub>, another critical cofactor for PLD activation [78, 112, 115].

Roles for PLD/PA/DAG signaling in the myocardium, particularly in myocardial protection, have been suggested. Like the kinase pathways activated during I/R and serving protective functions [67, 116–119], there is considerable evidence that oxidative stress activates and regulates PLD activity [120–126]. Activation of PLD in response to oxidative stress is associated with various cardiac pathologies, including coronary heart diseases [127–129]. PLD has been reported to be involved in cardioprotection by ischemic preconditioning, a phenomenon in which brief episodes of I/R render the myocardium insensitive to a subsequent prolonged ischemic episode [130, 131]. Pharmacologically induced activation of PLD was shown to reduce infarct size, while inhibition of PLD blocked the beneficial effects of preconditioning in isolated rabbit and rat hearts [130, 131]. Interestingly, adenosine-induced protection against I/R injury was suggested to be mediated through RhoA and a direct interaction with PLD1, as it was blocked by a mutant PLD1

that did not bind RhoA [132]. Thus, PLD1 activity appears to be involved in cardioprotection, although mechanisms for its activation and protective function have not been elucidated.

#### Protein Kinase Activation and DAG

As mentioned above, DAG generated through the actions of PLC or PLD activates PKC. PKC has been implicated in cardiac metabolism, contractile function, hypertrophy, heart failure, fibrosis, inflammation, and responses to ischemic injury. The predominant isozyme of PKC in the ventricle is PKC $\alpha$ , an isoform shown by Molkenin's group to play a role in heart failure susceptibility and cardiac contractility [133, 134]. The novel PKC isozymes, PKC $\epsilon$  and PKC $\delta$ , have been suggested to play divergent roles in I/R injury. PKC $\epsilon$  has been shown to confer cardioprotection against I/R injury and to contribute to the protective effects of preconditioning [98–100, 135] and postconditioning [101, 136] in various animal models and in the human myocardium [102]. While some data also implicate PKC $\delta$  in cardioprotection [137], most evidence suggests that PKC $\delta$  is proapoptotic and has detrimental effects in the heart [138–142]. In the setting of I/R injury, it has been shown that either PKC $\epsilon$  activation or PKC $\delta$  inhibition reduce I/R damage, whereas PKC $\epsilon$  inhibition or PKC $\delta$  activation increase injury [102, 140, 143]. Additionally, combined PKC $\epsilon$  activation and PKC $\delta$  inhibition have been shown to exert additive protection against I/R injury in isolated rat hearts [144].

#### Protein Kinase D Activation

Protein kinase D (PKD) is activated in the adult myocardium [145] and in other tissues through effects of novel PKCs [146–148]. There are a growing number of functions attributed to PKD signaling in the heart, including regulation of contractile function through phosphorylation of troponin-I [149, 150], and phosphorylation of HDAC-5, a class II HDAC, that regulates cardiac hypertrophy [151–153]. In other systems, PKD has also been shown to function, via nuclear factor  $\kappa$ B signaling, as a mediator of cell survival [154–156]. Notably, PKD activity has been reported to be regulated by RhoA. Expression of a constitutively activated RhoA increased basal PKD kinase activity in COS-7 cells [157] and induced PKD activation-loop phosphorylation in HeLa cells [158]. RhoA-induced PKD activation was suggested to be mediated through ROCK and PKC $\epsilon$  since treatment with the ROCK inhibitor Y-27632 or knockdown of PKC $\epsilon$  (but not PKC $\delta$ ) by siRNA inhibited RhoA-induced PKD phosphorylation in HeLa cells [158]. A Rho/ROCK/PKC signaling pathway has also been reported to be upstream of PKD-induced protection

against oxidative stress in intestinal epithelial cells [159]. A functional role of PKD for cardiac protection has not, to our knowledge, been demonstrated.

## RhoA and Gene Expression

### RhoA and SRF

Rho GTPases are best known for their role in regulation of cytoskeletal dynamics through effectors that control cell adhesion, morphology, and motility [160–169]. Rho GTPases also play a critical, albeit generally less appreciated role, in transcriptional regulation, as first noted based on RhoA-dependent regulation of serum response factor (SRF) target genes [170]. The SRF protein is constitutively localized to the nucleus and bound to serum response element (SRE) sequences, and no direct modifications of the protein are required for its function [171–174]. Rather SRF associates with other transcription factors to provide combinatorial control of its target genes [175]. Two major classes of coactivators, regulated by separate signaling pathways, are known to activate SRF: the ternary complex factors (TCFs) and the myocardin-related transcription factors (MRTFs) [176–179].

The TCF family is activated by MAP kinase-mediated phosphorylation [176, 179, 180]. However, Treisman's group showed that RhoA effects on SRF activity were mediated through a TCF independent pathway [170]. RhoA activation was also shown to stimulate *c-fos* SRE transcription in a TCF-independent manner [181]. In cardiomyocytes, we reported that RhoA affects ANF gene expression through TCF independent SRE sites [182]. Myocardin, MRTF-A and MRTF-B comprise the second, more recently characterized SRF coactivator family [179, 183, 184]. The activity of MRTF-A and MRTF-B depends on RhoA signaling and actin dynamics [179, 185–189]. Association of MRTF-A with G-actin results in its sequestration in the cytoplasm. Serum stimulation and other signals that activate RhoA promote actin polymerization [188, 190, 191], leading to MRTF-A translocation into the nucleus and SRF target gene activation [179, 188, 192]. Immediate-early genes, SRF itself, skeletal  $\alpha$ -actin, and myosin light chain-2 (MLC-2v), are among the genes regulated in this manner [180, 193–195]. Also notable among the SRF-regulated genes are the growth factor inducible immediate early genes CCN1 (Cyr61) and CCN2 (CTGF), which belong to the CCN family of matricellular proteins [196–199].

### RhoA and CCN1/Cyr61

CCN1 was first identified as an immediate early gene upregulated in response to growth factors and subsequently

determined to be secreted from the cell, where it serves a function intermediate between that of ECM proteins and growth factors [200–202]. CCN1 is a pleiotropic molecule, acting via cell surface integrin engagement to regulate cell migration, proliferation, and survival [197, 198, 203, 204]. Mechanical stretch induces CCN1 expression [205–207], and recent studies showed that MRTF-A and CREB binding proteins are required for mechanical strain-induced transcriptional activation of the CCN1 gene in vitro and in vivo [207, 208] (see Fig. 3). Mechanical overload-induced CCN1 gene expression in vivo was also associated with RhoA-mediated nuclear localization of MRTF-A and enrichment of SRE sites on the CCN1 promoter with MRTF-A and acetylated histone H3.

### CCN1 in the Heart

Little is known about the regulation or role of CCN1 in the heart, but several papers report that CCN1 expression is highly expressed in the myocardium of patients with heart failure or ischemic myopathy [209–211]. CCN1 expression has also been shown to increase in mouse heart in response to pressure overload and myocardial infarction and in cardiomyocytes stimulated by GPCR agonists [211]. Multiple signaling pathways including activation of ERK and PKC can contribute to induction of CCN1 expression [211]. In addition, there is considerable evidence that signaling through RhoA plays a major role in CCN1 induction in response to S1P and other agonists in cardiomyocytes (Zhao et al., manuscript in preparation) as in other cell types [204, 205, 208, 212, 213]. A paper by Yoshida et al. [214] provides intriguing evidence supporting the hypothesis that CCN1 is cardioprotective. These investigators observed that CCN1 addition to isolated cardiomyocytes attenuated the response to oxidative stress and that this occurred via CCN1 effects on integrin  $\beta$ 1-mediated FAK and Akt activation. Thus, RhoA-mediated increases in CCN1 expression and release are a potential mechanism by which the cell can further activate integrins, FAK, and protective Akt signals (see Fig. 3).

## Conclusion

One of the challenges faced by maturing scientists is that of remembering what we published and defended in the past and squaring it with our more recent findings and made by our colleagues. The solace is that the old theories advanced the field to the stage where they can now be revisited and revised using more sophisticated approaches. The notion that RhoA serves as a mediator of cardiac hypertrophy, one that we proposed and others espoused a decade ago, is not wrong, but the role of RhoA in this response appears minor



by comparison with that of other pathways shown to be essential and efficacious hypertrophic mediators. Pharmacologic inhibitors of ROCK have been developed in the last decade and have proven to be remarkable tools for further discovery, including demonstration that vascular tone is regulated by biochemically defined RhoA/ROCK pathways. These inhibitors have since been shown to be useful in treating a plethora of cardiovascular pathologies, from hypertension to atherosclerosis, and from heart failure to ischemic damage. While targets for the effects of ROCK inhibitors may be known, the cellular site of their action is not. Indeed, a notion we propose here is that whereas chronic RhoA signaling through ROCK may be a villain in inflammatory cells, fibroblasts, endothelial cells, and vascular smooth muscle, more acute activation of RhoA, at least within the cardiomyocyte, may serve to promote survival. There is evidence that RhoA is protective in a number of contexts, and there are multiple potential direct targets for RhoA that could mediate such responses. We suggest that RhoA is activated in the myocyte along with other protective pathways and that its effects on the cytoskeleton, phospholipids, or gene expression could be used to aid the ailing myocyte. If salutary pathways can be uncovered, they would be potential targets for cardioprotection. Accordingly, a prudent approach to treating conditions such as ischemic heart diseases might be to avoid the use of RhoA/ROCK inhibitors during the earliest phases of ischemic injury.

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