Hypotonic swelling-induced activation of PKN1 mediates cell survival in cardiac myocytes

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THE OSMOLARITY OF BODY FLUIDS is tightly regulated in mammalian tissues (23). However, in patients with impaired renal, hepatic, or cardiac function, excessive water ingestion or loss of electrolytes could cause a transient decrease in plasma osmolality and consequent cell swelling (57). Cell swelling not only causes dilution of intracellular contents but also induces deformation of the cell membrane and the underlying cytoskeletal network (46). This initiates changes in the activities of intracellular signaling molecules, thereby evoking a wide variety of cellular responses, from modulation of ion transporters/channels to changes in gene expression. Some of these swelling-induced cell responses are compensatory and physiological, but others are pathological (13, 16, 23). For example, in the heart, changes in the activities of ion transporters/channels could be electrogenic and cause fatal cardiac arrhythmia. This is most relevant in the context of myocardial ischemia and reperfusion, where both cell swelling and arrhythmias are commonly observed (26, 27). Thus, understanding the signaling mechanism activated by hypotonic swelling in mammalian cells is very important.

We (50) have previously shown that an increase in membrane tension caused by hypotonic stress rapidly activates tyrosine kinases, including Src, and subsequently activates members of the MAPK family, including ERK1/2. In budding yeast, a MAPK cascade that consists of Bck1, Mkk1/2, and Mpk1 is specifically activated by increases in cell wall stress, including low osmolality, and its components are called “cell integrity MAPKs” (for reviews, see Refs. 21 and 33). These kinases are structurally homologous to mammalian MEKK, MEK, and ERK, respectively. In fact, functional defects in yeast Bck1 mutants are rescued by MEKK1 (9), and mammalian ERK1 is able to stimulate a pathway normally controlled by Mpk1 in yeast (5). Although these results suggest that a hypotonic stress-activated MAPK cascade similar to the yeast cell integrity MAPK cascade may exist in mammalian cells, the nature of such a MAPK cascade has not been well characterized.

The yeast cell integrity MAPK cascade is regulated by Pkc1, a homolog of mammalian PKCs (14, 28, 31, 44). Pkc1 is activated by increases in cell wall stress, including hypotonic stress, and growth defects in mutants defective in Pkc1 can be partially suppressed by osmotic stabilization (32). Although the kinase domain of yeast Pkc1 is homologous to that of mammalian PKCs, we (50) have previously shown that hypotonic stress does not activate the conventional or novel isoforms of PKCs in cardiac myocytes. Thus, it is unknown whether the similarity between the aforementioned yeast and mammalian MAPK cascades exists at the level of yeast Pkc1.

PKN1 is a serine/threonine protein kinase with an NH2-terminal regulatory region highly homologous to that of yeast Pkc1 (36) and a catalytic domain homologous to that of PKC (42) (for a review, see Ref. 38). Pkc1 and PKN1 are both regulated by Rho family small GTP-binding proteins, Rho1 and RhoA, respectively (2, 29, 51, 58). Pkc1 and PKN1 are also both regulated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) (15, 18, 24). Both RhoA- and phosphatidylinositol 3′-kinase (PI3K)-dependent signaling mechanisms have been implicated in cellular responses initiated by hypotonic stress (55). Furthermore, PKN1 has been suggested as an upstream regulator of the MKK3/6-ERK6 pathway in mouse fibroblasts (35). Thus, PKN1 may be a nodal mammalian

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kinase that links cell swelling with the downstream specific MAPK cascade. However, it has not been tested whether mammalian PKN1 is activated by hypotonic stress in mammalian cells.

In this study, to elucidate the signaling mechanism activated by cell swelling in mammalian cells, we first investigated whether or not PKN1, a mammalian homolog of yeast Pkc1, is activated by hypotonic cell swelling. Second, we investigated whether RhoA and PDK1, upstream regulators of PKN1, are activated by hypotonic cell swelling. Finally, we examined whether the activation of PKN1 is able to stimulate downstream MAPKs. Finally, we examined whether the activation of PKN1 by hypotonic swelling affects cardiac myocyte survival.

**MATERIALS AND METHODS**

*Plasmids.* Constitutively active PKN1 [CA-PKN1; PKN1 (561-942)] (54) was generated by PCR and subcloned into pCR3.1 (Invitrogen), and the entire open reading frame was sequenced to confirm the correct sequence.

*Primary cultures of neonatal rat ventricular myocytes and application of hypotonic stress.* Primary cultures of cardiac ventricular myocytes from 1-day-old Crl:WI/BR-Wistar rats (Charles River Laboratories) were prepared as previously described (37). In brief, ventricular myocytes were dispersed from the ventricles by digestion with 0.1% collagenase type IV (Sigma), 0.1% trypsin (GIBCO-BRL), and 15 µg/ml DNase I (Sigma). Cell suspensions were applied to a discontinuous Percoll gradient as previously described (25). Cells were cultured in cardiac myocyte culture medium consisting of DMEM-F-12 supplemented with 5% horse serum, 4 g/ml aprotinin, 0.7 ng/ml sodium selenite (GIBCO-BRL), 2 g/l BSA (fraction V), 3 mM pyruvic acid, 15 mM HEPES, 100 µM ascorbic acid, 100 µg/ml ampicillin, 5 µg/ml linoelic acid, and 100 µM 5-bromo-2'-deoxyuridine (Sigma). We obtained cultures in which >95% of cells were myocytes, as assessed by immunofluorescence staining with a monoclonal antibody against sarcomeric myosin (MF20). Culture media were changed to serum-free media at 24 h before experiments. The osmolarity of the culture medium was changed by gentle replacement with prewarmed media having 67% osmolarity, as previously described (50). All experiments involving animals were approved by the Institutional Animal Care and Use Committee at the New Jersey Medical School.

*Immunoprecipitation and immunoblot analysis of PKN1.* For the immunoprecipitation of PKN1, myocytes were lysed with 500 µl lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Igepal CA-630, 0.1% SDS, 0.1 mM Na3VO4, 1 mM NaF, 0.5 mM 4-(2-aminoethyl) benzensulfonyl fluoride hydrochloride (AEBSF), 0.5 µg/ml aprotinin, and 0.5 µg/ml leupeptin. Cell debris was pelleted by centrifugation (15,000 rpm at 4°C for 20 min). Immunoprecipitation was carried out by the addition of 1 µg/ml anti-PKN1 antibody (Transduction Laboratories) for 2 h followed by the addition of 40 µl of protein G-Sepharose (50% slurry, Pharmacia Biotech) for 1 h at 4°C. Immunoprecipitates were washed three times with the lysis buffer without AEBSF and subjected to SDS-PAGE on 6.5% gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore) and probed with 1 µg/ml anti-PKN1 antibody (Transduction Laboratories) followed by horseradish peroxidase-conjugated protein G (Pharmacia Biotech) at 1:5,000 dilution. For immunoblot analyses, myocytes were lysed with 100 µl lysis buffer, and equal amounts of protein (50 µg) were used. Anti-Thr774 phosphorylated PKN1, anti-Ser241 phosphorylated PDK1, anti-PDK1 (Cell Signaling Technology), and anti-PKN1 (Transduction Laboratories) antibodies were used.

**PKN1 assay.** PKN1 was immunoprecipitated as described above and incubated with 25 µl of a reaction mixture containing 20 mM Tris-HCl (pH 7.5), 4 mM MgCl2, 20 µM ATP, and 0.8 µCi [γ-32P]ATP (together with 40 µM PKC-δ substrate (AMFPTMNRRG-SIKQAK) as a peptide substrate based on the PKC-δ substrate sequence (39). After an incubation at 30°C for 30 min, the reaction was stopped by spotting the supernatants onto Whatman P81 paper and submersion in 75 mM phosphoric acid. After three washes with 75 mM phosphoric acid, the incorporation of 32P into the PKC-δ peptide was measured by scintillation counting (39, 45).

**Subcellular fractionation.** Subcellular fractionization was performed as previously described (3). Cell-free lysates were prepared by the addition of 100 µl hypotonic lysis buffer (per 60-mm dish) containing 20 mM Tris (pH 8.0), 3 mM MgCl2, 0.4 mM AEBSF, 5 µg/ml aprotinin, 2 µg/ml trypsin inhibitor, and 20 µM leupeptin. After three cycles of freeze and thaw, samples were centrifuged at 100,000 g at 4°C for 60 min. The supernatant was saved as the “soluble” fraction. Pellets were washed twice with the same lysis buffer and resuspended in 100 µl lysis buffer supplemented with 1% (vol/vol) Triton X-100 and 0.1% SDS. Cell debris was separated by centrifugation (14,000 rpm at 4°C for 20 min), and the supernatant was saved as the “particulate” fraction. The protein content of each fraction was determined by the Lowry method.

**Immunoblot analysis of Rho proteins.** Cell lysate and immunoprecipitation of RhoA were performed as previously described (3). Either cell lysates containing equal amounts of protein or immunoprecipitates were subjected to SDS-PAGE on 16% gels. Proteins were transferred onto PVDF membranes and probed with 1 µg/ml anti-RhoA antibody (Santa Cruz Biotechnology) followed by horseradish peroxidase-conjugated anti-rabbit IgG (New England BioLab) at 1:6,000 dilution. The protein probes were visualized using the enhanced chemiluminescence system (Amersham) in the linear range of X-ray films.

**Preparation of recombinant proteins and in vitro GTP-RhoA binding assay.** The cDNA fragment encoding amino acid residues 3–135 of human PKN1, which contains the reported Rho-binding site (51), was generated by PCR and subcloned into the pGEX-4T-3 vector (Pharmacia Biotech) in frame. The recombinant NH2-terminal RhoA-binding domain of PKN1 (residues 3–135) was expressed as a glutathione-S-transferase (GST) fusion protein in Escherichia coli (BL21 Gold, Stratagene). GST-PKN1 (3–135) was purified by glutathione-Sepharose (Pharmacia Biotech) chromatography. Ventricular myocytes were exposed to hypotonic stress in serum-free medium. The medium was aspirated, and cells were washed three times with ice-cold PBS and extracted by scraping into 300 µl lysis buffer [20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 100 mM KCl, 5 mM MgCl2, 5 mM NaF, 0.2 mM Na3VO4, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 0.5% (vol/vol) 2-mercaptoethanol, 10 mM benzamidine, 0.2 mM leupeptin, 0.5 µg/ml aprotinin, and 0.5 µg/ml leupeptin (11)]. Lysates were centrifuged (10,000 g, 5 min, 4°C), and supernatants were assayed for protein by the Lowry method. Supernatants were incubated with mixing at 4°C for 3 h with GST-PKN1(3–135) bound to glutathione-Sepharose beads resuspended in the lysis buffer. Beads were washed four times with 1 ml lysis buffer and boiled with SDS-PAGE sample buffer. The eluted proteins were resolved on 16% SDS-PAGE gels, transferred to PVDF membranes, and probed with 1 µg/ml anti-RhoA antibody (Santa Cruz Biotechnology) followed by horseradish peroxidase-conjugated protein A (Zymed) at 1:10,000 dilution.

**Transient transfection and reporter gene assays.** For transient transfection, myocytes were plated at a density of 7 × 105 cells/well in six-well plates. Transfections were carried out with 10 µl Lipofectamine 2000 (GIBCO-BRL) in 1 ml/well of serum-free DMEM-F-12 medium as previously described (37). Atrial natriuretic factor (ANF)-luciferase (Luc) (~638, 1 µg), containing a 638-bp fragment of the ANF promoter linked to firefly luciferase, or pFR-Luc, containing 5 copies of the GAL4-binding site linked to firefly luciferase.
was used as a reporter gene. In the latter case, a mammalian expression plasmid encoding the GAL4 DNA-binding domain fused to the Elk1 activation domain was cotransfected. To evaluate the effect of the signaling molecules on activities of transcription factors, we cotransfected various amounts of mammalian expression plasmids encoding CA-PKN1, kinase inactive PKN1 (KI-PKN1), or dominant negative MEK1 (DN-MEK1) or the corresponding empty vectors. To correct for differences in the transfection efficiency, an simian virus 40-driven β-galactosidase construct (SV40-βgal; 0.5 μg) was cotransfected. Twenty-four hours after transfection, media were changed, and myocytes were cultured in serum-free conditions for an additional 24 h. Alternatively, hypotonic stress was applied for 3 h beginning 45 h after transfection. Myocytes were then lysed with 100 μl/well of Reporter lysis buffer (Promega), and luciferase activities were measured as previously described. Activities of β-galactosidase were determined using Lumigal 530 (Lumigen) (37).

Quantitative RT-PCR. Total RNA was prepared using the RNeasy fibrous tissue kit (QIAGEN), and first-strand cDNA was then synthesized using the ThermoScript RT-PCR system (Invitrogen). The following oligonucleotide primers were used in this study: ANF, sense 5'-TATTCCTTCGTGACCACAGCTGAACGT-3' and antisense 5'-ATCTTCGGTACCGGAAGCTG-3'; c-Kit, sense 5'-ATGGGCTCCTTCTCCATCAC-3' and antisense 5'-TAGGAA-CACGGAAGCCCATGCG-3'; skeletal actin, sense 5'-GATTGACATCATCCGATGTT-3' and antisense 5'-GATTGACATCATCCGATGTT-3'; and GAPDH, sense 5'-TTCTTGTGCAGTGCCAGCCTCG-3' and antisense 5'-TATTCCTTCGTGACCACAGCTGAACGT-3'.

**Construction of adenoviruses.** Recombinant adenovirus vectors were constructed, propagated, and titered as previously described (37). Briefly, pBHGloxΔE1,3Cre (Microbix), including the ΔE1 adenoviral genome, was cotransfected with the pDC shuttle vector containing the gene of interest into 293 cells using Lipofectamine 2000 (Invitrogen). Through homologous recombination, the test genes were integrated into the E1-deleted adenoviral genome. The viruses were propagated in 293 cells. We made replication-defective human adenovirus type 5 (devoid of E1) harboring full-length PKN1 (Ad-PKN1). Adenovirus harboring β-galactosidase (Ad-LacZ) was used as a control.

**Construction of short hairpin RNA adenoviral expression vectors.** The pSilencer 1.0-U6 expression vector was purchased from Ambion. The U6 RNA polymerase III promoter and polylinker region were subcloned into the adenoviral shuttle vector pDC311 (Microbix). The hairpin-forming oligonucleotide (5'-GATTGACATCATCCGATGTTCAAGAGACATGCGGATGATGTCAATC-3') of the rat PKN1 cDNA and its antisense primer with Apel and HindIII overhangs were synthesized, annealed, and subcloned distal to the U6 promoter (the loop sequence is underlined). A recombinant adenovirus was generated using homologous recombination in 293 cells as described above.

**Cell viability of the cells.** Cell viability of the cells was measured by Cell Titer Blue (CTB) assays (Promega). In brief, cardiac myocytes (1 × 10⁵ cells/100 μl) were seeded onto 96-well dishes. After 24 h, the media were changed to serum-free media. Cardiac myocytes were transduced with adenovirus harboring PKN1, DN-PKN, or LacZ for 24 h or short hairpin (sh)RNA against PKN1 or control shRNA for 72 h and then treated with hypotonic stress for 8 h. Viable cell number was determined using Lumigal 530 (Lumigen) (37).

**Results.**

**A:** PKN1 was immunoprecipitated and resolved on 6% gels. In lane 6, the substrate was phosphorylated PKN1. PKN1 was immunoprecipitated, and the enzyme activity was determined using the PKC-δ peptide as the substrate. The level of PKN1 kinase activity in the control state was designated as 1. Results are expressed as means ± SE obtained from 4–6 independent experiments. *P < 0.05 vs. control.

**B:** Electrophoretic mobility shift of proteins. The mobility of proteins was determined using the PKC-δ peptide as the substrate. The level of PKN1 kinase activity in the control state was designated as 1. Results are expressed as means ± SE obtained from 4–6 independent experiments. *P < 0.05 vs. control.

**C:** Cell lysates were subjected to immuno blot analyses with anti-Thr774 phosphorylated (p-)PKN1 and anti-total PKN1 antibodies.

**D:** Thr774 p-PKN1/total PKN1 was determined after densitometric analyses. The ratio at 60 min (hypotonic) was expressed relative to that at time 0 (control). E: Cell lysates were subjected to immunoblot analyses with anti-Ser221 p-phoshoinositide-dependent protein kinase-1 (PDK1) and anti-total PDK1 antibodies. F: Ser727 p-PDK1/total PDK1 was determined after densitometric analyses. The ratio at 60 min was expressed relative to that at time 0.
numbers were measured by Cell Titer Blue assays performed according to the supplier’s protocol. Experiments were conducted in triplicate at least three times.

Statistics. Data are given as means ± SE. Statistical analyses were performed using ANOVA. Significance was accepted at the P < 0.05 level.

RESULTS

Cardiac myocytes express PKN1 but not PKN2. Among the PKN family of protein kinases, PKN1 and PKN2 exhibit 87% amino acid identity, and both are regulated by Rho family small GTP-binding proteins. The results of immunoprecipitation and immunoblot analyses indicated that cardiac myocytes express PKN1, whereas PKN2 was not detectable (data not shown).

Activation and phosphorylation of PKN1 in response to hypotonic cell swelling. We examined whether hypotonic stress (67% osmolarity) activates PKN1 using immune complex kinase assays. Kinase activity of the immune complex toward the PKC-δ peptide was increased within 15 min of hypotonic stress, peaked at 60 min, and gradually decreased thereafter (Fig. 1A). The kinase activity in the control immunoprecipitate was <10% of that in the PKN1 immunoprecipitate and did not change in response to hypotonic stress. Activation of PKN1 was not caused by nonspecific mechanical disturbances due to medium change because application of the culture medium alone caused no significant changes in the kinase activity of PKN1 at 60 min (1.04 ± 0.07, n = 4). These results indicate that PKN1 is activated by hypotonic stress. In the rest of the study, hypotonic stress was applied using 67% osmolarity.

Increases in PKN1 kinase activity were accompanied by an electrophoretic mobility shift of PKN1 in the direction of higher molecular weights (Fig. 1B). The mobility shift of PKN1 occurred within 15 min of hypotonic stress and persisted for >2 h. It has been shown that a similar electrophoretic mobility shift of PKN1 occurs when PKN1 is phosphorylated by the PKN1 kinase PDK1 (15, 18), suggesting that hypotonic stress-induced PKN1 activation may be accompanied by the phosphorylation of PKN1. Immunoblot analyses with antibodies against Thr774 phosphorylated PKN1 and total PKN1 indicated that Thr774 phosphorylation of PKN1 was increased 1.5-fold at 60 min and 1.4-fold at 120 min in response to hypotonic stress (Fig. 1, C and D, and Supplemental Material, Supplemental Fig. S1).1 We also examined whether hypotonic stress activates PDK1. Cell lysates were probed with antibodies against Ser241 phosphorylated PDK1 and total PDK1. Quantitative analysis indicated that hypotonic stress increased Ser241 phosphorylation of PDK1 1.5-fold at 60 min (Fig. 1, E and F).

Hypotonic stress causes the translocation of RhoA from the soluble fraction to the particulate fraction. RhoA, a Rho family small GTP-binding protein, binds to the NH2-terminal regulatory region of PKN1 and activates PKN1 when it is bound by GTP (51). Yeast Rho1 is homologous to human RhoA, and the phenotype of the rho1Δ mutant is partially suppressed by the expression of human RhoA (47). Rh1 is activated by increased wall stress, including hypotonic stress, in yeast and controls signal transmission through Pkc1 (8, 29). RhoA is activated by hypotonic stress in human umbilical cord vein endothelial cells (22), and an exoenzyme C3-sensitive mechanism has been suggested to mediate hypotonic stress-induced anionic efflux (56). We therefore examined whether RhoA is activated by hypotonic stress in cardiac myocytes. Since RhoA translocates from the soluble fraction to the particulate fraction upon activation (3, 10), we examined the effect of hypotonic stress on the subcellular localization of RhoA. In control myocytes, the majority of RhoA was detected in the soluble fraction.
Hypotonic stress caused a rapid and significant increase in RhoA content in the particulate fraction (3.1 ± 0.9-fold increase at 15 min vs. control, \( P < 0.05 \); Fig. 2A and B).

To further confirm that RhoA is activated in response to hypotonic stress, we measured the specific in vitro interaction between the NH2-terminal Rho-binding domain of PKN1 (amino acid residues 3–135) and RhoA-GTP, an active form of RhoA (51). Cell extracts prepared from myocytes with or without hypotonic stress were incubated with GST alone or GST-PKN1 (3–135) immobilized on glutathione-Sepharose columns. Bound RhoA was eluted and quantitated by immunoblot analyses. Hypotonic stress significantly increased the binding of RhoA to GST-PKN1 (3–135), which reached a peak within 15 min (2.5 ± 0.2-fold increase at 15 min vs. control, \( P < 0.05 \)) and reverted to baseline by 60 min (Fig. 2C). The time course of RhoA binding to GST-PKN1 (3–135) was similar to that of RhoA translocation to the particulate fraction. No significant binding of RhoA was observed when myocyte extracts were incubated with GST alone (data not shown). Since only the GTP-bound form of RhoA interacts with PKN1, this result also suggests that hypotonic stress activates RhoA.

The Src family of tyrosine kinases mediates hypotonic stress-induced activation of RhoA, PDK1, and PKN1. We (50) have previously shown that hypotonic stress rapidly (within 5 s) activates tyrosine kinases, including Src, in cardiac myocytes. Our preliminary results indicated that the activation of Src is inhibitory and that the activation of RhoA is regulatory.

A.

**Peptide kinase activity (fold)**

**Hypotonic Stress (min)**

- **PP1 (-)**
- **PP1 (+)**

B.

**Relative phospho-PKN1**

- **LacZ**
- **DN-Src**
- **LacZ**
- **DN-Src**

C.

<table>
<thead>
<tr>
<th>Hypotonic Stress (min)</th>
<th>Soluble fraction</th>
<th>Particulate fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>PP1 (-)</td>
<td>PP1 (+)</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
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<tr>
<td>15</td>
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D.

**Time (min)**

- **PP1 (10 \( \mu \)M)**

**RhoA (particulate fraction)**

- **Time (min)**

- **PP1 (10 \( \mu \)M)**

Fig. 3. Effects of PP1, a selective Src family tyrosine kinase inhibitor, on hypotonic cell swelling-induced RhoA-PKN1 activation. Myocytes were pretreated with PP1 (10 \( \mu \)M) for 60 min, and hypotonic stimulation (67% osmolarity) was then applied for the indicated times. A: PKN1 kinase activity was determined by a peptide kinase assay using the PKC-\( \beta \)-peptide as the substrate, which was performed after immuno-precipitating PKN1 with an anti-PKN1 antibody. The level of PKN1 kinase activity in the control state was designated as 1. Results are expressed as means ± SE obtained from 3 independent experiments. PKN1 kinase activities of immunoprecipitates without pretreatment with PP1 (■) and with pretreatment with PP1 (○) are shown. *\( P < 0.05 \) vs. cells without hypotonic stress. B, left: cardiac myocytes were transduced with adenovirus harboring LacZ or dominant negative Src (DN-Src) for 48 h, and hypotonic stimulation (67% osmolarity) was then applied for 60 min. Cell lysates were subjected to immunoblot analyses with anti-Thr774 p-PKN1, anti-total PKN1, and anti-tubulin antibodies. Right, Thr774 p-PKN1/total PKN1 was determined after densitometric analyses. The value in LacZ-treated samples without hypotonic stress was expressed as 1. The results were obtained from 4 experiments.

C, left: cardiac myocytes were stimulated with hypotonic media (67% osmolarity) for the indicated times and fractionated into soluble (lanes 1–3) and particulate (lanes 4–6) fractions. Equal amounts of protein were loaded in each lane. Immunoblot analyses were performed using specific anti-RhoA antibody. The results shown are representative of 4 independent experiments. *\( P < 0.05 \) vs. cells without hypotonic stress. D, in vitro interactions of the recombinant NH2-terminal Rho-binding domain of PKN1 [GST-PKN1 (3–135)] with RhoA were evaluated by an in vitro binding assay. Equal amounts of protein were loaded in each lane, and immunoblot analyses were performed using specific anti-RhoA antibody. Bound RhoA was detected by elution of the complex with glutathione and blotting for RhoA. In lanes 4–6, the experiments were conducted in the presence of PP1 (10 \( \mu \)M). The results shown are representative of 3 independent experiments.
required for hypotonic stress-induced c-fos expression in mouse fibroblasts (50). Src family tyrosine kinases have been implicated as important regulators of RhoA. We therefore examined the role of Src in hypotonic stress-induced RhoA-PKN1 activation. PP1 (10 μM), a selective Src tyrosine kinase inhibitor (30), significantly suppressed hypotonic stress-induced PKN1 activation (Fig. 3A) and phosphorylation (Fig. 4, A and C), and hypotonic stress-induced increases in PKN1 phosphorylation were inhibited in the presence of dominant negative Src (Fig. 3B). PP1 also significantly inhibited hypotonic stress-induced increases in RhoA content in the particulate fraction (Fig. 3C) and in the in vitro interaction between the NH2-terminal Rho-binding domain of PKN1 and RhoA (Fig. 3D). Taken together, these results suggest that hypotonic stress-induced activation of RhoA and PKN1 is mediated by Src.

Inhibition of Src by PP1 also suppressed the hypotonic stress-induced activation of PDK1 (Fig. 4, A and B). Hypotonic stress-induced phosphorylation of both PDK1 and PKN1 was similarly abolished in the presence of the PI3K inhibitor wortmannin (Fig. 4, A–C). Thus, Src mediates hypotonic stress-induced activation of PKN1 through PDK1-dependent mechanisms as well. Taken together, these results suggest that the two major regulators of PKN1, RhoA and PDK1, and, therefore, PKN1 itself are critically regulated by Src during hypotonic stress.

CA-PKN1 activates ERKs in mammalian cells. In yeast, activation of Rho1 and Pck1 by increased wall stress stimulates a downstream MAPK cascade comprised of Bck1, Mkk1/Mkk2, and Mpk1 (29, 33). We (50) have previously shown that hypotonic stress strongly activates ERK1/2. Mammalian ERK can substitute for a signaling mechanism normally controlled by Mpk1 in Saccharomyces cerevisiae (5). We therefore examined whether CA-PKN1 (54) activates the MEK-ERK pathway in cardiac myocytes. Since the efficiency of plasmid transfection is modest in cardiac myocytes, we used sensitive reporter gene assays to detect the ERK-dependent phosphorylation of Elk1-GAL4. Cotransfection with CA-PKN1 significantly increased transcriptional activity of Elk1-GAL4 (2.8 ± 0.6-fold increase vs. control, P < 0.05; Fig. 5A). Activation of Elk1-GAL4 induced by CA-PKN1 was significantly suppressed in the presence of DN-MEK1, suggesting that the MEK-ERK pathway mediates CA-PKN1-induced Elk1-GAL4 activation. Hypotonic stress for 3 h activated Elk1-GAL4 1.6-fold. This hypotonic stress-induced activation of Elk1-GAL4 was inhibited in the presence of ki-PKN1, suggesting that the activation of PKN1 plays an important role in mediating the hypotonic stress-induced activation of Elk1 (Fig. 5B).

To confirm that PKN1 activates ERK in cardiac myocytes, cardiac myocytes were transduced with adenovirus harboring PKN1 (Ad-PKN1) or LacZ (Ad-LacZ, control) and cultured in serum-free conditions. The level of phosphorylated ERK1/2 was significantly increased in myocytes transduced with Ad-PKN1 compared with those with Ad-LacZ (Fig. 5C), suggesting that PKN1 induces the activation of ERK1/2.

To examine the role of endogenous PKN1 in mediating hypotonic stress-induced activation of ERK, we prepared adenovirus harboring shRNA-PKN1. We confirmed that the expression of PKN1 is significantly attenuated in myocytes transduced with Ad-shRNA-PKN1 (data not shown). Phosphorylation of ERK1/2 was significantly attenuated in myocytes transduced with Ad-shRNA-PKN1 both at baseline and after hypotonic stress (Fig. 5D). These results suggest that PKN1...
plays an essential role in mediating the activation of ERK1/2 in response to hypotonic stress in cardiac myocytes.

Activation of PKN1 protects cardiac myocytes from hypotonic stress-induced cell death. To evaluate the functional significance of the activation of the PKN1-ERK pathway under hypotonic stress, we tested the effect of PKN1 knockdown and ERK inhibition upon the viability of cardiac myocytes under hypotonic stress. Hypotonic stress significantly reduced the viability of cultured cardiac myocytes. Suppression of PKN1 and ERK activation by shRNA knockdown of PKN1 or U-0126, a selective inhibitor of the ERK1/2 and ERK5 cascades, significantly reduced further the survival of cardiac myocytes in response to hypotonic stress (Fig. 6, A and B). These results suggest that the activation of the PKN1-ERK pathway plays an essential role in mediating protection against cell death under hypotonic stress.

DISCUSSION

Hypotonic stress activates PKN1 and its upstream regulators, RhoA and PDK1, in cardiac myocytes, with a mechanism similar to the hypotonic stress-activated signaling mechanism in yeast. While this signaling complex activates the Bck-Mkk-Mpk pathway in yeast, the similar MEK-ERK signaling pathway is activated in cardiac myocytes. Such remarkable parallelism may be helpful in identifying the remaining components of the hypotonic stress signaling mechanism in mammalian cells, such as initial osmo-sensing mechanisms and downstream targets of the MAPK cascade.

Yeast Pkc1 is regulated by multiple upstream signaling molecules, including Rho1 (29), PDK1 (24), and Cdc28-Cln1/2 (20). Mammalian PKN1 is also regulated by multiple signaling molecules, including Rho (19) and PDK1 (15, 18). We have shown that hypotonic stress activates RhoA and increases its binding to the NH2-terminal HR1 domain of PKN1 in vitro. Hypotonic stress also activates PDK1 and Thr774 phosphorylation of PKN1. A comparison of the time course of activation suggested that the activation of RhoA reached a peak (15 min) earlier than the activation of PDK1 or the mobility shift of PKN1 (60 min) in response to hypotonic stress. Since precedent association of PKN1 with RhoA is required for interactions between PKN1 and PDK1, the activation of PKN1 may be mediated by sequential assembly with upstream regulators and subsequent phosphorylation of PKN1 by PDK1. The activity of PKN1 returns to baseline by 120 min despite the continued presence of PKN1 phosphorylation when the activity of RhoA comes back to normal. We speculate that RhoA and PDK1 synergistically enhance hypotonic stress-induced activation of PKN1 and that dephosphorylation of PKN1 takes place with a slower time course than inactivation of PKN1. Both RhoA (41, 55, 56) and PI3K-dependent (7, 17, 34, 59) mechanisms have been implicated in osmolarity sensing, possibly representing cell adhesion-mediated and membrane lipid-mediated mechanisms, respectively. Regulation by multiple upstream regulatory mechanisms may allow myocytes to finely regulate PKN1 activities.
extracellular matrix (12, 48), and mechanical deformation at focal adhesions cause the activation of RhoA (1), elucidating the involvement of these mechanisms in hypotonic stress-induced RhoA activation is also of great interest.

We (49) have previously shown that Src family tyrosine kinases are rapidly (within 5 s) activated in cardiac myocytes. Since Src is activated more rapidly than RhoA and PKN1 in response to hypotonic stress, and because hypotonic stress-induced activation of RhoA and PKN1 is blocked in the presence of a Src-specific inhibitor, it is likely that Src is an upstream regulator of RhoA and PKN1 in the hypotonic signaling cascade. Tyrosine kinases may be involved in the activation of RhoA by heterotrimeric G proteins (40, 43). It should be noted that yeast does not possess tyrosine-specific protein kinases (21). Thus, diversity between mammalian and yeast signaling mechanisms may exist at the level of mammalian Src. Alternatively, there may exist a Src-equivalent (but not tyrosine-specific) protein kinase in yeast that regulates either yeast Rho1 and/or PDK1 in response to hypotonic stress.

PKN1 phosphorylates MLK-like mitogen-activated protein triple kinase (MLTK), thereby stimulating the downstream MKK3/6-ERK6 (p38\textsuperscript{Y}) protein kinase cascade (35, 53). Since ERK1/2 are the predominant MAPKs activated by hypotonic stress (50) and since human ERK1 has been shown to mimic the function of yeast Mpk1 (5), ERK1/2 seem to represent terminal kinases in the MAPK cascade activated by hypotonic stress. Our results suggest that, whereas wild-type PKN1 activates ERK1/2, downregulation of PKN1 abolishes the hypotonic stress-induced activation of ERK1/2. Thus, we believe that the ERK pathway is most likely regulated by endogenous PKN1. PKN1 regulation of the MEK-ERK pathway is homologous to the yeast cascade, where Pck1 regulates the downstream Mkk-Mpk pathway.

Hypotonic stress induced in the heart during ischemia and reperfusion causes sarcolemmal blebs, osmotic fragility, and, eventually, cell death (4). The role of the swelling-activated signaling mechanisms in the regulating survival and death of cardiac myocytes is not well understood. Cardiac swelling-activated Cl\textsuperscript{−} current (I\textsubscript{Cl,swell}) plays an important role in mediating arrhythmogenesis, myocardial injury, preconditioning, and apoptosis. Although the persistent activation of I\textsubscript{Cl,swell} in dilated cardiomyopathy may be protective by limiting the prolongation of action potential duration, I\textsubscript{Cl,swell} also induces apoptosis and is therefore detrimental (6). Our results suggest that hypotonic swelling-induced activation of the PKN1-ERK pathway acts as an adaptive mechanism to alleviate cellular injury and death.

We (52) have recently shown that transgenic mice with cardiac-specific overexpression of CA-PKN exhibit mild cardiac hypertrophy. However, adenovirus-mediated transduction of PKN in cultured cardiac myocytes did not induce obvious increases in cell size, at least acutely (Supplemental Fig. S2). Although we believe that the hypotonic stress-induced activation of PKN1 is primarily an adaptive mechanism, further investigation is needed to elucidate whether or not PKN affects swelling and/or regulation of cell volume.

In summary, hypotonic stress activates PKN1, accompanied by the activation of RhoA and PDK1, and increases in PKN1 activity lead to the activation of the downstream MEK-ERK pathway. We propose that cardiac myocytes possess a hypotonic stress-activated signaling cascade similar to the yeast cell...
wall stress-activated signaling mechanism. This pathway may potentially be involved in transcriptional and growth control of cardiac myocytes in response to hypotonic stress and ischemia-reperfusion in the heart. The elucidation of upstream regulators and downstream targets of this signaling cascade should dramatically increase our understanding of how mammalian cells adapt in response to osmotic disturbances and subsequent increases in membrane tension caused by cell swelling.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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