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# The Rho effector, PKN, regulates ANF gene transcription in cardiomyocytes through a serum response element

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**Morrisette, Michael R., Valerie P. Sah, Christopher C. Glembotski, and Joan Heller Brown.** The Rho effector, PKN, regulates ANF gene transcription in cardiomyocytes through a serum response element. *Am J Physiol Heart Circ Physiol* 278: H1769–H1774, 2000.—The low-molecular-weight GTP-binding protein RhoA mediates hypertrophic growth and atrial natriuretic factor (ANF) gene expression in neonatal rat ventricular myocytes. Neither the effector nor the promoter elements through which Rho exerts its regulatory effects on ANF gene expression have been elucidated. When constitutively activated forms of Rho kinase and two protein kinase C-related kinases, PKN (PRK1) and PRK2, were compared, only PKN generated a robust stimulation of a luciferase reporter gene driven by a 638-bp fragment on the ANF promoter. This ANF promoter fragment contains a proximal serum response element (SRE) and an Sp-1-like element required for the transcriptional response to phenylephrine (PE). This response was inhibited by dominant negative Rho. The ability of dominant negative Rho to inhibit the response to PE and the ability of PKN to stimulate ANF reporter gene expression were both lost when the SRE was mutated. Mutation of the Sp-1-like element also attenuated the response to PKN. A minimal promoter driven by ANF SRE sequences was sufficient to confer Rho- and PKN-mediated gene expression. Interestingly, PKN preferentially stimulated the ANF versus the *c-fos* SRE reporter gene. Thus PKN and Rho are able to regulate transcriptional activation of the ANF SRE by a common element that could implicate PKN as a downstream effector of Rho in transcriptional responses associated with hypertrophy.

signal transduction; hypertrophy; cardiac

THE LOW-MOLECULAR-WEIGHT GTPase Rho is a well-established regulator of actin cytoskeletal organization in fibroblasts (25, 30). In addition, a role for Rho in regulating *c-fos* gene expression has been reported (5, 7, 9, 41). The effects of Rho on *c-fos* gene expression in NIH 3T3 cells are mediated through the *c-fos* serum response element (SRE) and occur in a ternary complex factor (TCF)-independent manner.

Among the multitude of putative Rho effectors that have been cloned are a number of protein kinases that bind to and are activated by Rho (reviewed in Ref. 42).

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Rho kinase (ROK), which is the most extensively characterized Rho effector, was shown to regulate Rho-dependent cytoskeletal rearrangement. Transcriptional activation of the *c-fos* SRE by Rho was originally suggested to be mediated through ROK, but several lines of evidence from recent studies using a pharmacological ROK inhibitor Y-27632 and Rho effector mutants argue against ROK involvement in SRE-dependent transcription (5, 8, 13, 14, 19, 20, 32, 33). Other less well-characterized protein kinases that are activated by Rho are protein kinase N (PKN), also called protein kinase C-related kinase 1 (PRK1) (2, 46), and its closely related family member PRK2 (44). PKN and PRK2 share significant homology to PKC family members in their catalytic domains, but their amino terminal regulatory domains are distinct (22, 26). PKN is ubiquitously expressed in human tissues (23) and has been shown to interact with cytoskeletal proteins (24) and to increase glucose transport in adipocytes (37). A role for PKN in cytoskeletal or transcriptional regulation has not been clearly documented.

Rho has been implicated as a mediator of hypertrophic signaling in neonatal rat ventricular myocytes (3, 10, 12, 31, 38). In this well-characterized cultured cell model of hypertrophy, G<sub>q</sub>-coupled receptor agonists stimulate the expression of *c-fos* and other immediate early genes, promote reexpression of embryonic genes, including atrial natriuretic factor (ANF), and upregulate embryonic isoforms of contractile protein genes such as myosin light chain (MLC)-2v (4). We have shown that Rho is required for increases in ANF and MLC-2 expression mediated by  $\alpha_1$ -adrenergic receptor stimulation and that activated Rho can induce myofibrillar organization and increase myocyte size (12, 31). Rho function is also necessary for angiotensin II-stimulated myofibrillar changes and ANF induction (3) as well as G $\alpha_q$ -regulated ANF gene expression (10, 31). Activated Rho was also recently shown to stimulate transcriptional activation of the TCF-independent *c-fos* SRE in cardiomyocytes (41).

The ANF promoter contains two SRE-like cis elements that appear to function in a TCF-independent fashion (36), since they do not contain the flanking Ets motifs responsible for TCF binding. These SRE-like elements, along with Sp-1 sites, are required for conferring maximal  $\alpha_1$ -adrenergic receptor-stimulated ANF reporter gene expression (36). The experiments pre-

sented here tested the hypothesis that Rho regulates ANF expression through the ANF SRE. In addition we examined the ability of putative Rho effectors, ROK, PRK1, and PKN, to transcriptionally activate ANF reporter gene expression and determined whether regulation occurred at the level of the SRE.

## EXPERIMENTAL PROCEDURES

**Plasmids and reporter constructs.** Five reporter genes in which the 5'-flanking sequence of the rat ANF promoter or SRE consensus sequences were fused to firefly luciferase cDNA were used in this study: ANF-638, ANF-134, ANF134c114, 3xANF SRE, and 3x*c-fos* SRE. The 638-bp promoter (ANF-638) includes the 5'-flanking sequence from ANF -638 to +65 fused to luciferase, whereas the 134-bp promoter (ANF-134) includes ANF -134 to +65. Truncation of ANF-638 has been shown to confer maximal stimulation compared with the full-length promoter (36). The ANF-134 contains one SRE from ANF -117 to -108 with the sequence CTTTAAAGG. This promoter was mutated at the SRE to C TTGCCCTG (mutations underlined), rendering the SRE nonfunctional (ANF134c114). In addition, three ANF SRE sequences, ACTGATACTTTAAAGGGCATCT, or three *c-fos* SRE sequences, **CAGGATGTCCATATTAGGACATCT** (SREs underlined), linked in tandem behind a minimal ANF promoter consisting of ANF -65 to +65 (ANF 65) and fused to luciferase cDNA were utilized (39). Importantly, it should be noted that the three *c-fos* SRE sequences include a TCF-binding (Ets) domain (Ets motif in bold) flanking the SRE, whereas the three ANF SRE sequences, previously shown to act in a TCF-independent manner, lack a flanking Ets motif.

Other constructs tested here were obtained from the following sources. Dominant negative Rho (N19Rho) was provided by Dr. Gary Bokoch (The Scripps Research Institute). Constitutively activated PKN and Rho kinase plasmids were provided by Drs. Yoshitaka Ono (Kobe University) and Kozo Kaibuchi (Nara Institute of Science and Technology, Japan), and constitutively activated PRK2 was provided by Dr. Jeffrey Settleman (Massachusetts General Hospital Cancer Center and Harvard Medical School). All constitutively activated mutants of these proteins had deletions of their regulatory domains, leaving their catalytic domains intact.

**Cell culture.** Neonatal rat ventricular myocytes (NRVM) were prepared from 2- to 3-day-old Sprague-Dawley rat pups as described previously (15, 35). The ventricles were collagenase digested and myocytes purified over a Percoll gradient. The cells were plated on six-well plates coated with 1% gelatin and cultured overnight in a 4:1 mixture of Dulbecco's modified Eagle's medium and medium 199, supplemented with 10% horse serum, 5% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin.

**Transient transfections.** Transfection of NRVM was carried out using a modified calcium phosphate method as previously described (35). Briefly, six-well plates with each well containing 3 ml of serum-containing media were transfected with a total of 6.6 µg of plasmid DNA (4.8 µg of cDNA and 1.8 µg of luciferase reporter plasmid). After transfection, cells were washed and maintained for 48 h in serum-free media with or without  $\alpha_1$ -adrenergic receptor agonist PE (100 µM + 2 µM propranolol to block  $\beta$ -adrenergic receptors). Reporter gene activity was determined by lysing cells in buffer containing 1% Triton X-100, and luciferase activity was quantified using a Berthold luminometer as described (28). Luciferase data were normalized to protein concentration determined by Bradford analysis. The data were not normalized to  $\beta$ -galactosidase ( $\beta$ -Gal), because we have found that most agonists and

kinases, including PE and activated PKN (PKN\*) activate the cytomegalovirus (CMV) or SV40 promoters, which drive the  $\beta$ -Gal constructs; thus  $\beta$ -Gal activity does not reflect transfection efficiency. Although  $\beta$ -Gal was not routinely used, the relative responses to stimuli of various promoters tested here were the same when normalized to protein as when normalized to  $\beta$ -Gal in the assays where it was used. Each experiment was performed in triplicate, and the data in the figures represent a minimum of three experiments. Data were normalized to the maximal response (established using 100 µM PE) to pool separate experiments.

## RESULTS

**Activated PKN and PRK2 but not Rho kinase stimulate ANF gene expression.** Constitutively activated forms of putative Rho effectors were transiently expressed in NRVM, and their effects on ANF-luciferase gene expression were examined. A Rho kinase mutant, made constitutively active by removal of the NH<sub>2</sub>-terminal regulatory domain, did not stimulate ANF-638-luciferase reporter gene expression in NRVM (Fig. 1). Constitutively active forms of the protein kinase C-related kinases PRK2 and PKN (also known as PRK1), generated by the removal of their respective regulatory domains, were also examined for their ability to stimulate ANF-638-luciferase expression. Activated PKN gave a robust stimulation equivalent to ~60% of that induced by the well-established hypertrophic agonist PE ( $P < 0.01$ ). The stimulation by PRK2 was significant ( $P < 0.01$ ) but modest compared with that seen with PKN (Fig. 1).

**Rho-mediated ANF expression requires a functional SRE.** Because Rho effects on *c-fos* gene expression are mediated via the SRE, we examined the requirement for the SRE in Rho- and PKN-mediated transcriptional activation of the ANF gene. For these studies we tested both the ANF-638 promoter, which contains two SRE sites, as well as the truncated ANF-134 promoter, which contains only one SRE-like sequence. We also utilized the same promoters in which the SRE was mutated to render it nonfunctional (ANF638c114 and

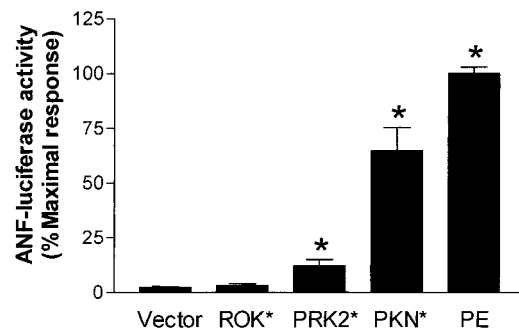


Fig. 1. Activated protein kinase N (PKN) and protein kinase C-related kinase 2 (PRK2) but not Rho kinase (ROK) stimulate atrial natriuretic factor (ANF) gene expression. Myocytes were transfected with ANF-638 luciferase reporter gene and appropriate empty vector or constitutively activated kinase (ROK\*, PRK2\*, PKN\*) and incubated in the presence or absence of 100 µM phenylephrine (PE). PE response was designated as 100%, and data in each experiment were normalized to their respective PE responses. Each bar represents mean  $\pm$  SE from at least 3 experiments performed in triplicate. \* $P < 0.01$  compared with vector.

ANF134c114). These constructs have been used previously to demonstrate the dependence of PE-stimulated ANF expression on these proximal SREs (36, 39). A dominant negative form of Rho was tested for its ability to block responses to PE. The data shown in Fig. 2 are expressed as a percentage of the response to PE to facilitate pooling of experiments in which the absolute effect of PE varied. For ANF-638, N19Rho inhibited the response to PE by ~70%; the response on ANF-134 to PE was inhibited by ~60% (Fig. 2A). In contrast N19Rho did not significantly inhibit PE activation of the ANF134c114 or ANF638c114 promoters lacking a SRE (Fig. 2B).

*A functional SRE is needed for transcriptional activation mediated by PKN.* The involvement of the proximal cis SRE element in transcriptional activation of the ANF gene by PKN was also examined. As shown in Fig. 3, the ANF-134 promoter retains responsiveness to PKN. However, when the proximal SRE site in either the ANF-638 or ANF-134 promoters is mutated to render it nonfunctional, the stimulation by activated PKN is markedly inhibited (ANF638c114) or lost (ANF134c114). These data demonstrate the requirement for the SRE in transcriptional activation of the ANF promoter by PKN (Fig. 3). Because the mutation

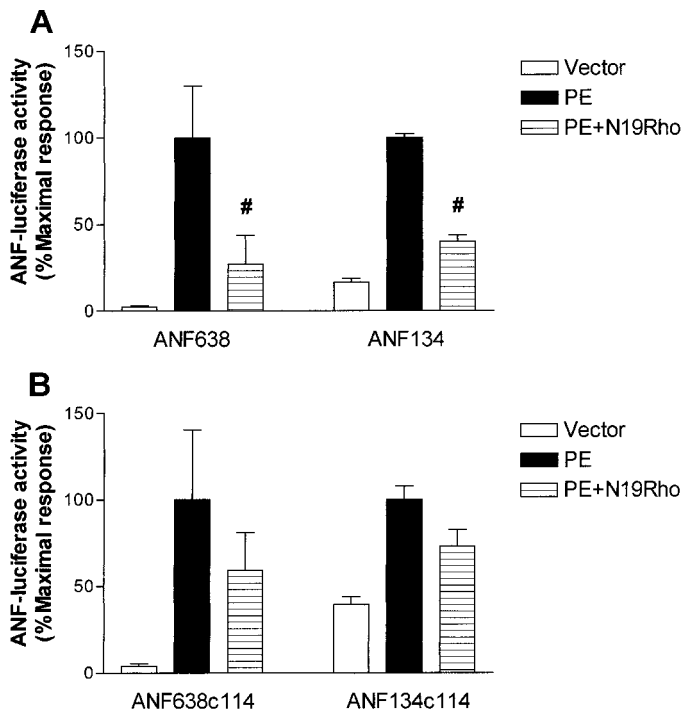


Fig. 2. Rho-mediated ANF expression and transcriptional activation by PKN requires a functional serum response element (SRE). Myocytes were transfected with the designated luciferase reporter gene (A: ANF-638, ANF-134; B: ANF638c114, ANF134c114) and empty vector or dominant negative Rho (N19Rho) and incubated in the absence or presence of 100  $\mu$ M PE. PE response was designated as 100%, and data in each experiment were normalized to the respective PE response. Absolute fold stimulation by PE (over vector control) was as follows: 62-fold for ANF-638; 21-fold for ANF638c114; 7-fold for ANF-134; and 3-fold for ANF134c114. Each bar represents mean  $\pm$  SE of data from at least 3 experiments performed in triplicate. # $P < 0.01$  compared with vector + PE.

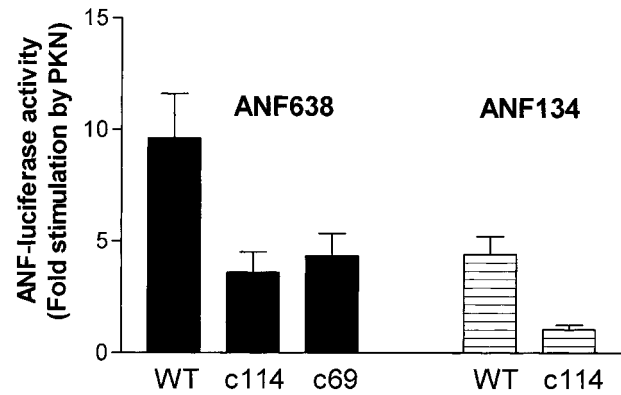


Fig. 3. Activated PKN regulates transcriptional activation of ANF expression through both SRE and Sp-1 elements. Myocytes were transfected with ANF-638, ANF638c114, ANF638c69, ANF-134, or ANF134c114-luciferase reporter gene and empty vector (not shown) or activated PKN. Results are shown as fold stimulation by PKN over empty vector control. Each bar represents mean  $\pm$  SE of data from at least 3 experiments performed in triplicate.

of the SRE in ANF-638 did not fully prevent the response to PKN, we examined the involvement of the Sp-1 site, which, like the SRE, has been shown to be important in PE-mediated ANF stimulation (36). Mutation of the Sp-1 site at -69 in the ANF-638 reporter gene (ANF638c69) resulted in a ~55% loss in stimulation by PKN, demonstrating that the Sp-1 site is also involved in transcriptional activation of ANF by PKN.

*Rho and PKN regulate transcriptional activation of ANF SRE and c-fos SRE.* To obtain further evidence that the SRE derived from the ANF gene could mediate the Rho-dependent effects of PE and the response to PKN, we used reporter gene constructs in which three copies of the SRE derived from the ANF promoter (3xANF SRE) were linked in tandem behind an ANF minimal promoter. Constructs containing three copies of the SRE from the *c-fos* promoter were also examined (39). PE induced a robust activation of the 3xANF SRE as well as the 3xc-*fos* SRE reporter genes. Significant (>40%) inhibition of both PE-stimulated ANF and *c-fos* expression was achieved by coexpression of N19Rho (Fig. 4A). PKN also stimulated the 3xANF SRE and 3xc-*fos* SRE luciferase. Interestingly, the fold stimulation of 3xANF SRE by PKN was ~80% of that induced by PE (Fig. 4B), whereas the stimulatory effect of PKN on 3xc-*fos* SRE luciferase expression was modest compared with that of PE (Fig. 4B).

## DISCUSSION

The low-molecular-weight GTPase Rho has been shown to regulate diverse cellular functions. First identified as a regulator of actin stress fiber and focal adhesion complex formation (30), it has since been implicated in modulating gene expression, mitogen-activated protein (MAP) kinase activation, cell cycle progression, transformation, and smooth muscle contraction (9, 29, 33, 43). A role for Rho in myofibrillar organization and gene expression associated with myocardial cell hypertrophy has been demonstrated by our lab and others (3, 10, 12, 19, 31, 38, 41, 45).

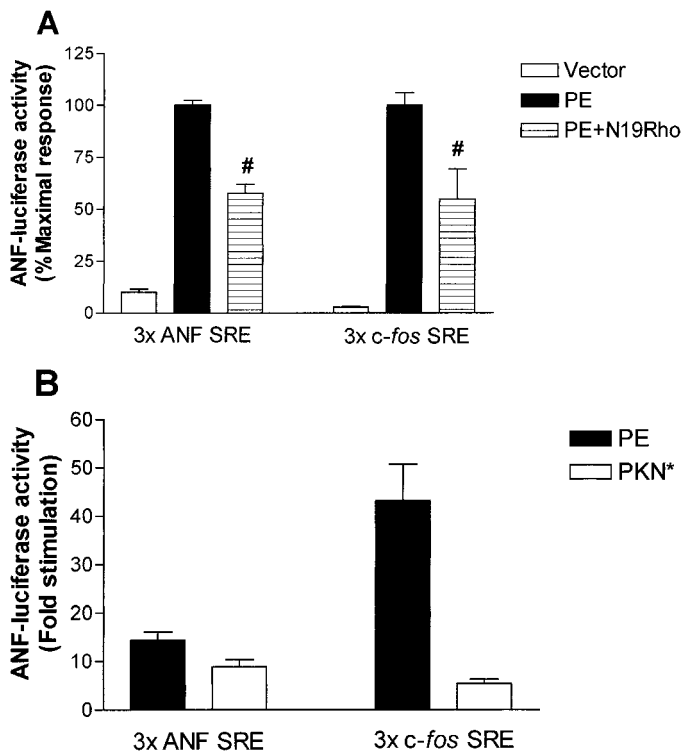


Fig. 4. Rho and PKN regulate transcriptional activation of ANF SRE and *c-fos* SRE. Cardiac myocytes were transfected with either ANF SRE or *c-fos* SRE luciferase reporter genes, empty vector, dominant negative Rho (N19Rho) (A), or PKN\* (B) in the absence or presence of 100  $\mu$ M PE. PE response was designated as 100%, and data in each experiment were normalized to the respective PE response in A. Results for B are shown as fold stimulation by PKN over empty vector control. Each bar represents mean  $\pm$  SE of data from at least 3 experiments performed in triplicate. # $P < 0.02$  compared with vector + PE.

Over the past few years numerous Rho effectors have been isolated and cloned. Currently, a question of great interest is how these effectors carry out the various and diverse functions of Rho. The best characterized of these effectors is Rho kinase, which has been convincingly shown to regulate cytoskeletal rearrangement (1, 5, 14, 20) and smooth muscle contraction (16–18, 34, 40, 43). We showed that three distinct Rho kinase mutants were able to attenuate Rho-stimulated myofibrillar organization and ANF expression in cardiomyocytes (12). Notably, responses to activated Rho and PE were most fully abolished by an interfering mutant consisting of the Rho-binding domain, which could also inhibit interactions of Rho with other effectors. Thus it was suggested that Rho signals through other effectors in addition to Rho kinase to elicit these hypertrophic responses.

To further examine the involvement of other Rho effectors, we compared the ability of three Rho-regulated kinases to stimulate ANF expression. The finding that constitutively activated Rho kinase failed to stimulate ANF reporter gene expression was surprising because the same constitutively activated mutant was previously reported to cause transcriptional activation of the *c-fos* SRE in fibroblasts (5). Rho kinase regulation of the *c-fos* SRE may be cell-type specific

because Rho was found to regulate MAP kinase activation in some cell types (29) but not others (6, 21), including cardiac myocytes (31). Recent reports using Rho effector mutants and Y-27632, a Rho kinase inhibitor, are consistent with our data and argue against a role for Rho kinase in SRE-mediated transcriptional regulation (32, 33). However, we recently found that a selective dominant negative ROK mutant is able to block PE-induced stimulation of the ANF-638 (M. R. Morissette, unpublished observations). (32). Endothelin-1 (ET-1)-mediated ANF gene expression in cardiomyocytes was also recently shown to be Rho kinase-dependent based on the use of the pharmacological inhibitor compound, Y-27632. Notably the responses to ET-1 and Rho kinase were independent of the SRE (19), thus Rho kinase may play a role in mediating PE and ET-1-induced ANF transcription through response elements other than the SRE.

The PKC-related kinases PKN and PRK2 were also examined. Previously published data indicated that PRK2 can act in concert with Rho to potentiate the effects of Rho on SRE transcriptional activation (27). In NRVM, both PKN and PRK2 stimulated ANF luciferase gene expression above vector control; however, PKN was significantly more efficacious in this regard (Fig. 1). This same stimulation pattern was seen using the ANF-134, 3xANF SRE, and 3xc-*fos* SRE in addition to the ANF-638 (data not shown). We cannot rule out the possibility that this selectivity is the result of differences in expression levels of the two protein kinases, because attempts to assess expression levels are compromised by the low transfection efficiency of the NRVMs. Nonetheless, it is clear that expression of activated PKN can induce ANF luciferase gene expression.

That the stimulatory effect of PE on the ANF-134 promoter is attenuated but not fully abolished by disruption of the SRE site suggested that additional elements in the 134-bp fragment (e.g., Sp-1) sites are responsive to PE (36). Studies using ANF-638 indicate that mutation of the proximal SRE results in incomplete inhibition of the response to PKN and that mutation of the Sp-1 site also reduces PKN stimulation (Fig. 3). These data support the notion that similar to previously published data using PE, the control of ANF transcription by PKN may utilize Sp-1 as well as SRE sites.

Rho has been shown to activate the *c-fos* SRE via SRF in a TCF-independent manner (9, 41). The ANF SRE has also been hypothesized to be activated by SRF independent of TCF (36). Interestingly, whereas PKN robustly activated 3xANF SRE-driven luciferase expression, PKN was a relatively modest activator of 3xc-*fos* SRE relative to PE. The reason for this apparent selectivity of PKN for the ANF versus the *c-fos* SRE is unknown but may result from the lack of an Ets motif adjacent to the ANF SRE. Because TCF apparently does not bind to this region of the ANF gene (36, 39) PKN may be a more effective activator of SRE-mediated gene expression in its absence. Additionally, there is a slight difference in the core sequences of the

ANF and *c-fos* SREs; in the *c-fos* gene the SRE is a consensus match (CC[A/T]6GG), whereas in the ANF gene the SRE has one mismatch. This mismatch, which is known to slightly reduce the affinity of the SRE for SRF (11), could also contribute to the preferential stimulation of the ANF SRE by PKN.

The data in this paper suggest that transcriptional regulation of the ANF gene by Rho and one of its effectors PKN occurs at the level of the SRE. Thus Rho and PKN appear to share a distal end point in ANF gene regulation. Experiments using a putative dominant negative form of PKN (data not shown) have not provided evidence that this enzyme is a direct mediator of the physiological response to PE or Rho. Multiple kinase pathways, including PKC, MAP kinases, and calcium/calmodulin kinase, have been implicated in the regulation of the ANF promoter. Thus it is not surprising that dominant negative Rho or PKN does not fully inhibit PE-stimulated ANF luciferase expression and that other signaling molecules besides Rho are involved in transducing PE-evoked signals to the SRE. Nonetheless we establish for the first time that the Rho effector PKN modulates cardiac gene expression and demonstrate that PKN has a striking effect on SRE-mediated ANF gene expression in cardiac myocytes.

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