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Evidence for Direct Regulation of Myocardial Na^+/H^+ Exchanger Isoform 1 Phosphorylation and Activity by 90-kDa Ribosomal S6 Kinase (RSK): Effects of the Novel and Specific RSK Inhibitor fmk on Responses to α_1-Adrenergic Stimulation

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ABSTRACT

Multiple stimuli of physiological and pathophysiological significance, including α_1-adrenoceptor agonists, stimulate the cardiac sarcolemmal Na^+/H^+ exchanger isoform 1 (NHE1) through activation of the mitogen-activated or extracellular signal-regulated kinase (ERK) kinase (MEK) ERK-90-kDa ribosomal S6 kinase (RSK) signaling cascade. However, the individual contributions of ERK and RSK, which can each phosphorylate the NHE1 regulatory domain, to such stimulation are unknown. In the present study, we have used the novel RSK inhibitor fmk to determine the role of RSK as a direct regulator of NHE1 phosphorylation and activity in response to α_1-adrenergic stimulation, in ventricular myocytes isolated from the adult rat heart. Initial experiments confirmed that pretreatment of myocytes with fmk before exposure to the α_1-adrenoceptor agonist phenylephrine inhibited RSK C-terminal kinase activity and thereby RSK N-terminal kinase activation, without affecting MEK or ERK activation. Pretreatment of myocytes with fmk also inhibited phenylephrine-induced increases in NHE1 phosphorylation and the rate of NHE1-mediated H^+ efflux under conditions of intracellular acidosis. These findings reveal, for the first time to our knowledge, that RSK is the principal regulator of NHE1 phosphorylation and activity after α_1-adrenergic stimulation in adult myocardium.

Cohen et al. (2005) have recently described the synthesis and characterization of novel halomethylketone pyrrol pyrimidine inhibitors targeted at the 90-kDa ribosomal S6 kinase ($p90^{RSK}$ or RSK), whose rational design takes advantage of the unique presence of threonine and cysteine residues at two defined positions (Thr493 and Cys436) within the active site of the C-terminal kinase (CTK) domain of the enzyme. These "selectivity filters" allow one of the prototypical compounds, fmk, to irreversibly inhibit the CTK activity of RSK isoforms RSK1 and RSK2 with remarkable specificity (Cohen et al., 2005).

One putative cellular substrate for RSK is the ubiquitously expressed Na^+/H^+ exchanger isoform 1 (NHE1). Indeed, studies in NHE1-deficient fibroblasts complemented with wild-type or mutated NHE1 by stable transfection have shown that RSK-mediated phosphorylation of Ser703 in the NHE1 regulatory domain facilitates serum-induced stimulation of exchanger activity (Takahashi et al., 1999). It is noteworthy that in myocardial tissue and cells, NHE1 activity has been causally associated with both physiological processes [e.g., intracellular pH regulation (Leem et al., 1999); inotropic consequences of stretch (Alvarez et al., 1999) and neurohormonal stimuli, such as endothelin 1 (Kramer et al., 1991), angiotensin II (Matsui et al., 1995), and α_1-adrenergic agonists (Gambassi et al., 1992)] and pathological responses.

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ABBREVIATIONS: RSK, 90-kDa ribosomal S6 kinase; CTK, C-terminal kinase; NHE1, Na^+/H^+ exchanger isoform 1; ARVM, adult rat ventricular myocyte; NHE, Na^+/H^+ exchanger; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase or extracellular signal-regulated kinase kinase; GST, glutathione transferase; aa, amino acid; BCECF, 2′,7′-bis-(carboxyethyl)-5(6′)-carboxyfluorescein; HA, hemagglutinin; Adv, adenovirus, m, mouse; h, human; PFU, plaque-forming unit; DMSO, dimethyl sulfoxide; pH_i, intracellular pH; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid; NTK, N-terminal kinase; PDK, phosphoinositide-dependent kinase; IB, immunoblot/immunoblotting; PHEN, phenylephrine; CONT, control.

Relevant pathological responses include ischemia- and reperfusion-induced injury (for recent reviews, see Avkiran, 2001; Avkiran and Marber, 2002) and the development of hypertrophy and failure in various settings (Yoshida and Karmazyn, 2000; Chen et al., 2001; Engelhardt et al., 2002; Ennis et al., 2003; Aker et al., 2004). In this context, we have reported previously that, in adult rat ventricular myocytes (ARVMs), multiple (patho)physiologically relevant stimuli, such as α_1-adrenergic agonists (Snabaitis et al., 2000), angiotensin II (Gunasegaram et al., 1999), oxidative stress (Snabaitis et al., 2002), and sustained intracellular acidosis (Haworth et al., 2003), increase the activity of the sarcolemmal NHE (which is encoded by the NHE1 gene). Furthermore, the same stimuli induce coincident activation of the mitogen-activated or extracellular signal-regulated kinase (ERK) kinase (MEK)-ERK-RSK cascade (Gunasegaram et al., 1999; Snabaitis et al., 2000, 2002; Haworth et al., 2003). It is noteworthy that upstream inhibition of this cascade by targeting MEK also inhibits the stimulation of sarcolemmal NHE activity (Gunasegaram et al., 1999; Snabaitis et al., 2000, 2002; Haworth et al., 2003), strongly suggesting a critical NHE1-regulatory role for one or more components of the pertinent signaling pathway. However, to date, the individual contributions of ERK (which also can directly phosphorylate the NHE1 regulatory domain; Moor et al., 2001) versus its downstream effector RSK to the regulation of sarcolemmal NHE activity have not been delineated, principally because of the absence of selective inhibitors (Roberts et al., 2005).

In the present study, we have characterized the efficacy and specificity of the irreversible RSK inhibitor fmk in ARVMs and subsequently used this novel agent to determine the role of RSK as a direct regulator of NHE1 phosphorylation and sarcolemmal NHE activity in this cell type, in response to α_1-adrenergic stimulation.

Materials and Methods

Materials. Antibodies were from Cell Signaling Technology Inc. (Danvers, MA) (phospho-Ser 14-3-3 protein binding motif, phospho-Thr202/Tyr204 ERK, phospho-Ser386 RSK, and phospho-Thr577 RSK), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (agarose-conjugated RSK and NHE1), or BD Transduction Laboratories (Lexington, KY) (RSK). The bacterial expression vector pGEX-3X encoding the glutathione transferase (GST)-NHE1 fusion protein made up of aa 625 to 815 of human NHE1 was constructed using cDNA generated by polymerase chain reaction from pGEX-KG encoding a GST-NHE1 fusion protein made up of aa 516 to 815 of human NHE1 (a kind gift from Dr. Bradford Berk, University of Rochester Medical Center, Rochester, NY). The fluoroprobe 2′,7′-bis-(carboxyethyl)-5(6′)-carboxyfluorescein (BCECF), in membrane-permeant acetoxymethyl ester form, was from Calbiochem (San Diego, CA). The RSK inhibitor fmk was synthesized as described previously (Cohen et al., 2005). All other chemicals were from Sigma-Aldrich (St. Louis, MO) or VWR (West Chester, PA), unless otherwise stated.

Isolation, Culture, and Adenoviral Infection of ARVMs. ARVMs were isolated and maintained in culture for 18 h, with or without adenoviral infection shortly after isolation, as described previously (Snabaitis et al., 2005, 2006). Cells to be used for biochemical experiments were maintained in prelaminated six-well plastic culture dishes in modified M199 medium (Invitrogen, Carlsbad, CA), containing 2 mM creatine, 2 mM carnitine, and 5 mM taurine. Cells to be used for pH_i imaging were plated onto prelaminated glass coverslips and maintained in an identical medium, in 24-well plastic culture dishes.

Where indicated, adenoviral infection was performed, as described previously (Snabaitis et al., 2005), using newly constructed vectors encoding N-terminally HA-tagged wild-type mouse RSK2 (AdV-mRSK2) or C-terminally HA-tagged wild-type human NHE1 (AdV-hNHE1). The HA-tagged mouse RSK2 and human NHE1 constructs were kind gifts from Dr. Jeffrey A. Smith (University of Virginia, Charlottesville, VA) and Dr. Larry Fliegel (University of Alberta. Edmonton, AB, Canada), respectively, and the AdEasy system components were a kind gift from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). The RSK2 and NHE1 constructs were subcloned into the shuttle vector pAdTrackCMV and recombinant adenoviruses constructed by homologous recombination with the pAdEasy-1 adenoviral backbone vector in BJ5183 strain of *Escherichia coli*, as described by He et al. (1998). The recombinant adenoviruses were amplified in human embryonic kidney-293 cells and purified over CsCl gradients, which produced high-titer viral stocks of $>1.6 \times 10^{10}$ plaque-forming units (PFU)/ml. ARVM were infected with AdV-mRSK2 or AdV-hNHE1 at a multiplicity of infection of 50 PFU/cell 90 min after initial plating, and the adenovirus-containing medium was removed 60 min later and replaced with fresh modified M199 medium. Myocytes were maintained in an incubator (37°C; 5% CO_2) until used for experiments.

Pharmacological Treatment Protocols. ARVMs were pretreated for ≥90 min with 3 μM fmk or vehicle (0.03% DMSO) in the incubator, before being exposed to 10 or 100 μM phenylephrine, in the presence of 1 μM atenolol, for 3 min. In experiments that involved pH_i imaging, the latter treatment occurred on the microscope stage, during a transient (3-min) exposure to 20 mM NH_4Cl to induce intracellular acidosis (see below). For biochemical experiments, all treatments were carried out in the incubator, and ARVMs were lysed in either Laemmli sample buffer (for immunoblotting) or immunoprecipitation lysis buffer (for immunoprecipitation) at the end of the treatment period.

Western Immunoblotting. Western immunoblotting was carried out as described previously (Snabaitis et al., 2005, 2006). In brief, protein samples were separated by 7.5 to 12% SDS-PAGE, transferred to polyvinylidene difluoride or nitrocellulose membranes, and probed with appropriate primary antibodies. Primary antibodies were detected by donkey anti-rabbit or sheep anti-mouse secondary antibodies linked to horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Specific protein bands were detected by enhanced chemiluminescence (GE Healthcare), and phosphorylation status was quantified on a calibrated densitometer (GS-800; Bio-Rad Laboratories, Hercules, CA) using Quantity One software, version 4.5.1 (Bio-Rad).

Preparation of Recombinant NHE1 Fusion Protein. Recombinant GST-NHE1 fusion protein was prepared as described previously (Roberts et al., 2005). In brief, the bacterial expression vector pGEX-3X encoding aa 625 to 815 of human NHE1 N-terminally linked to GST was transformed into BL21 strain of *E. coli*. Cultures were grown to sublog phase and induced with 0.5 mM isopropyl-β-D-thiogalctopyranoside. Cells were harvested and resuspended in phosphate-buffered saline containing 1% (v/v) Triton X-100 and Complete Mini protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and the GST-NHE1 fusion protein purified at 4°C by affinity chromatography using glutathione-Sepharose 4B columns (GE Healthcare).

Analysis of RSK N-Terminal Kinase Activation in ARVMs. ARVMs were lysed by the addition of ice-cold immunoprecipitation lysis buffer at pH 7.4, containing 50 mM Tris-HCl, 2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride 1 plus 1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μg/ml pepstatin, and scraped off the culture plate on ice. Cell lysates were then centrifuged at 14,000*g* for 30 min at 4°C, and the supernatants were incubated with 10 μg of RSK antibody, purchased as an agarose conjugate (Santa Cruz Biotechnology, Inc.), for 2 h at 4°C. The beads were washed four times with ice-cold lysis buffer and 4 times with ice-cold assay buffer at pH 7.4,

containing 30 mM Tris-HCl, 15 mM $MgCl_2$, and 1 mM dithiothreitol. The immune complex was then incubated with 50 μl of reaction mixture containing 0.1 mM unlabeled ATP and 100 pmol of GST-NHE1 fusion protein as substrate; this reaction mixture was made up in assay buffer at pH 7.4, as described above. The reaction was allowed to proceed for 15 min at 37°C and terminated by the addition of Laemmli sample buffer. Proteins were resolved by 12% SDS-PAGE and GST-NHE1 phosphorylation status analyzed by Western immunoblotting using a phospho-Ser 14-3-3 protein binding motif antibody, which recognizes RSK-phosphorylated sites in the NHE1 regulatory domain (Snabaitis et al., 2006).

Analysis of Endogenous or Heterologously Expressed NHE1 Phosphorylation in ARVMs. Phosphorylation status of endogenous and heterologously expressed NHE1 was determined using an adaptation of a method that we have described recently (Snabaitis et al., 2006). ARVMs (uninfected or infected with AdV-hNHE1) were washed with ice-cold phosphate-buffered saline and lysed in lysis buffer at pH 7.5 containing 50 mM Tris-HCl, 5 mM EGTA, 2 mM EDTA, 100 mM NaF, and 1 mM Na_3VO_4 as well as 0.05% digitonin and Complete Mini protease inhibitor cocktail (Roche Diagnostics). The samples were then frozen by floating the culture plate on a volume of liquid N_2 and thawed at room temperature. Subsequently, cell lysates were centrifuged at 14,000*g* for 30 min at 4°C, and the supernatant was discarded. The pellet was then solubilized in ice-cold immunoprecipitation lysis buffer at pH 7.5 containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 100 mM NaF as well as 1% Triton X-100, 0.1% SDS, and Complete Mini protease inhibitor cocktail (Roche Diagnostics). The samples were centrifuged at 14,000*g* for 60 min at 4°C, after which the supernatant containing the solubilized membranes was removed and incubated overnight at 4°C with mouse monoclonal phospho-Ser 14-3-3 protein binding motif antibody. Immune complexes were mixed with protein A magnetic beads (New England Biolabs, Ipswich, MA) for 2 h at 4°C, washed three times with ice-cold modified immunoprecipitation lysis buffer not containing SDS, and separated using a magnetic separation rack (New England Biolabs). The immune complexes were dissociated by the addition of Laemmli sample buffer and heated for 5 min at 70°C. Protein samples from crude lysate or the immune complex were resolved on 7.5% SDS-PAGE and analyzed by Western immunoblotting using rabbit polyclonal NHE1 antibody.

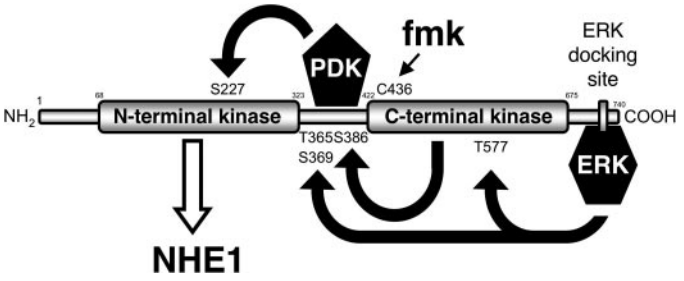

Fig. 1. An illustration of the key regulatory features of RSK (amino acid numbering refers to the RSK2 isoform). RSK contains two active domains, a CTK and an NTK. In the currently accepted model of RSK regulation, ERK associates with a docking site near the C terminus (Smith et al., 1999) and phosphorylates Thr577 in the activation loop of the CTK and Thr365/Ser369 in the linker region (Dalby et al., 1998; Frödin and Gammeltoft, 1999). Activation of the CTK then leads to autophosphorylation of Ser386, also in the linker region, which creates a docking site for PDK1 and facilitates PDK1-mediated phosphorylation of Ser227 in the activation loop of the NTK (Jensen et al., 1999; Frödin et al., 2000). The active NTK then phosphorylates the cellular substrates of RSK, such as NHE1. The specific RSK inhibitor fmk inhibits CTK activity by interacting with a reactive Cys residue (Cys436) within the active site of the CTK domain, with this interaction facilitated by the presence of a Thr gatekeeper (Thr493) flanking the hydrophobic pocket (Cohen et al., 2005).

Determination of Sarcolemmal NHE Activity in ARVMs. Sarcolemmal NHE activity was measured in quiescent ARVMs, as described previously (Yasutake et al., 1996; Avkiran and Yokoyama, 2000; Snabaitis et al., 2000), with the exception that the dual excitation/single emission pH-sensitive fluoroprobe BCECF was used, in conjunction with an imaging system (IonOptix, Milton, MA). In brief, ARVMs cultured on laminin-coated glass coverslips were placed in a cell chamber (model RC-25F; Warner Instruments, Hamden, CT) on the stage of an inverted microepifluorescence microscope (Nikon Eclipse TE300, equipped with a Nikon Plan Fluar 10×/0.3 numerical aperture objective), loaded with 2 μM BCECF-acetoxymethyl ester (15 min) and superfused (3 ml/min; 34°C) with HCO_3^--free Tyrode's solution at pH 7.4 containing 10 mM HEPES, 137 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, and 1.0 mM $CaCl_2$. Cells were excited with light at 440 and 495 nm using a HyperSwitch light source (IonOptix), with the BCECF fluorescence emission acquired from multiple cells simultaneously at 535 nm using a Dage-MTI (Michigan City, IN) charge-coupled device integrating video camera and IonWizard version 4.4 software (IonOptix). Calibration of the fluorescence signal was carried out, as described previously (Yasutake et al., 1996; Avkiran and Yokoyama, 2000; Snabaitis et al., 2000), using modified calibration solutions at pH 5.8 to 8.0 containing 140 mM KCl, 2 mM EGTA, 10 mM MES, PIPES, or HEPES, 1.2 mM $MgSO_4$, 0.001 mM

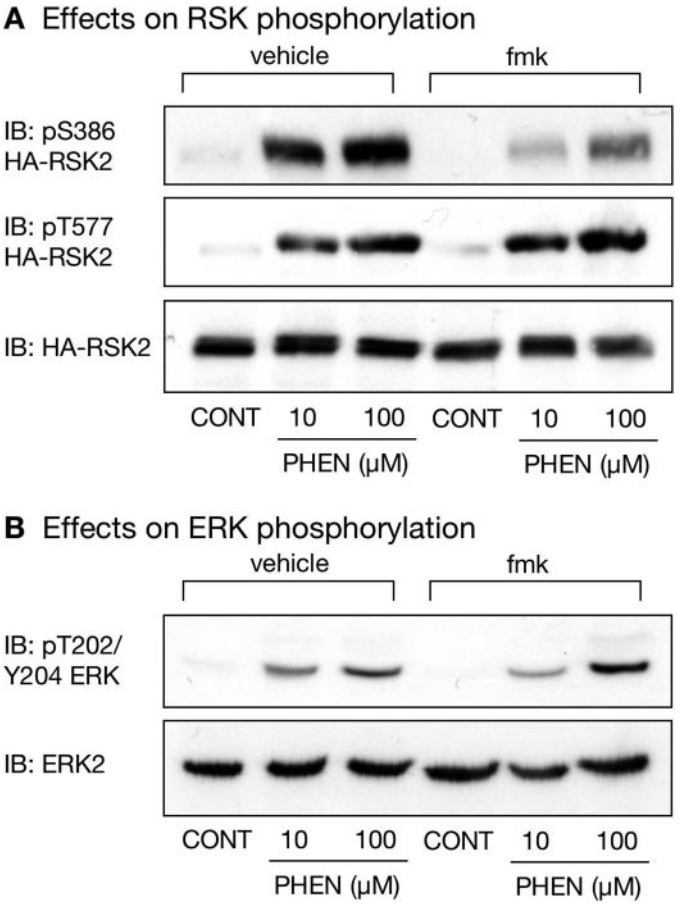

Fig. 2. Effects of fmk pretreatment on α_1-adrenoceptor-mediated activation of the RSK CTK (as reflected by HA-RSK2 phosphorylation at Ser386) and ERK (as reflected by HA-RSK2 phosphorylation at Thr577) (A), and MEK (as reflected by ERK phosphorylation at Thr202/Tyr204), in ARVMs with heterologous expression of HA-RSK2 (B). Cells were pretreated with 3 μM fmk or vehicle (0.03% DMSO) for 90 min before being exposed to phenylephrine (PHEN) at 10 or 100 μM for 3 min. RSK or ERK phosphorylation status was determined by Western immunoblotting (IB) with appropriate phospho-specific antibodies. The autoradiograms shown are representative of at least three independent experiments.

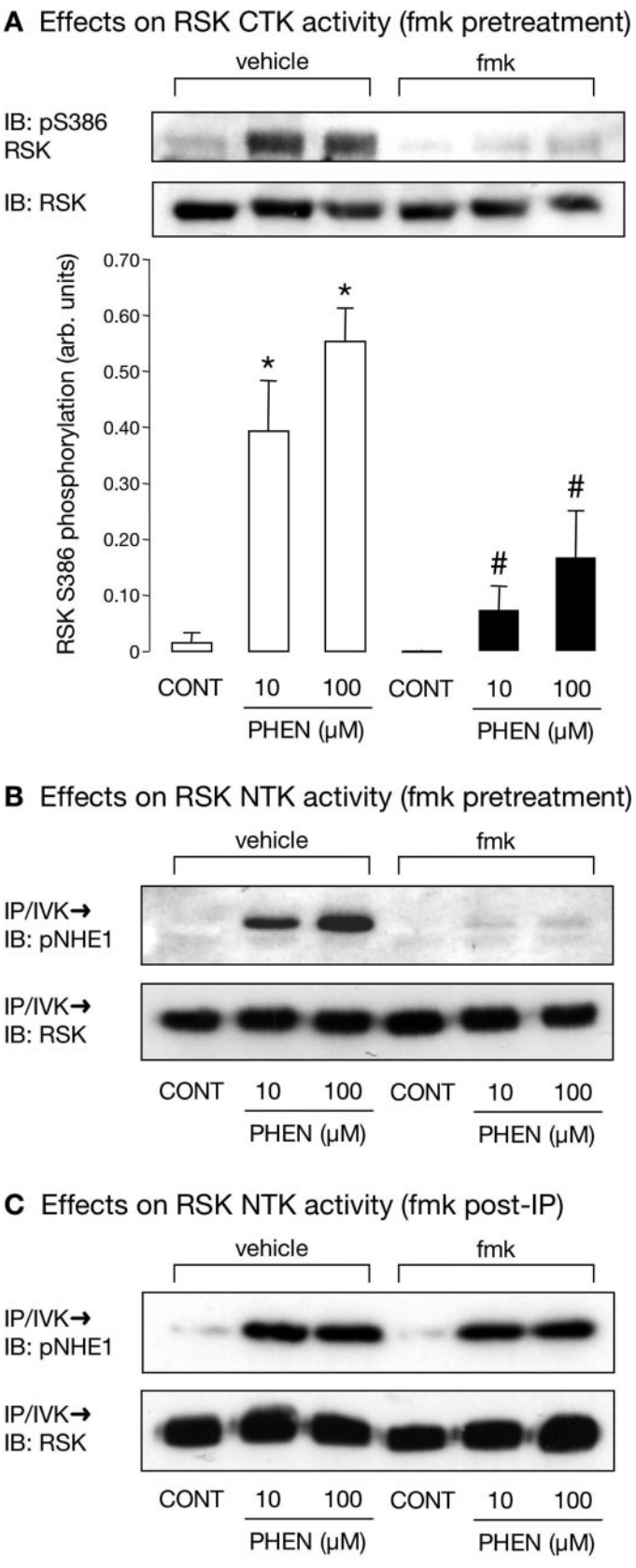

Fig. 3. Effects of fmk pretreatment on α_1-adrenoceptor-mediated activation of the RSK CTK (as reflected by endogenous RSK phosphorylation at Ser386, detected by Western IB analysis using a phospho-Ser386 RSK antibody) (A), and the RSK NTK [as reflected by phosphorylation of a GST-NHE1 fusion protein by immunoprecipitated RSK in an in vitro kinase (IVK) assay, detected by Western IB analysis using a phospho-Ser 14-3-3 protein binding motif antibody), in ARVMs (B). Cells were pretreated with 3 μM fmk or vehicle (0.03% DMSO) for 90 min before being exposed to PHEN at 10 or 100 μM for 3 min. In C, a similar in vitro kinase assay to that in B was used, with the exception that 3 μM fmk was only added to the reaction mixture after exposure of ARVMs to phenylephrine and RSK immunoprecipitation. The autoradiograms shown are representative of at least three independent experiments. The bar chart shows quantitative data (mean ± S.E.M.) from five independent experiments. *, $P < 0.05$ versus appropriate control (CONT) group; #, $P < 0.05$ versus corresponding vehicle-treated group.

carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 0.001 mM valinomycin, 0.01 mM nigericin, and 10 mM glucose. Cells were exposed transiently (3 min) to 20 mM NH_4Cl to induce intracellular acidosis, and the rate of acid efflux (J_H) measured during subsequent recovery from intracellular acidosis was used as the index of sarcolemmal NHE activity (Yasutake et al., 1996; Avkiran and Yokoyama, 2000; Snabaitis et al., 2000).

Statistical Analysis. Quantitative data are mean ± S.E.M. Student's *t* test was used to compare two groups. Otherwise, data were subjected to analysis of variance, with further analysis by Student-Newman-Keuls test (for multiple comparisons).

Results

Figure 1 illustrates the cascade of events involved in RSK activation in the cellular context as well as the proposed site of action of fmk. In initial experiments, we induced heterologous expression of an epitope-tagged mouse RSK2 construct (HA-RSK2) in ARVMs, using the recombinant adenovirus AdV-mRSK2; this approach allowed robust responses to be readily detected by phospho-specific antibodies that recognize RSK when phosphorylated at Ser386 (an autophosphorylation target for the RSK CTK) or Thr577 (a specific target for upstream ERK), after α_1-adrenoceptor stimulation. Exposure of vehicle-treated ARVMs to the α_1-adrenoceptor agonist phenylephrine at 10 or 100 μM induced marked increases in the phosphorylation of both Ser386 and Thr577 in HA-RSK2 (Fig. 2A), reflecting increased cellular activity of the RSK CTK and ERK, respectively. Pretreatment of ARVMs with 3 μM fmk attenuated the increase in Ser386 phosphorylation, particularly in response to 10 μM phenylephrine, but it had no inhibitory effect on the increase in Thr577 phosphorylation (Fig. 2A), indicating inhibition of the RSK CTK but not ERK activity. The phenylephrine-induced increase in the dual phosphorylation of ERK at Thr202/Tyr204 (targets of upstream MEK) was unaffected by fmk pretreatment (Fig. 2B), indicating the absence of an inhibitory effect on MEK activity.

Having confirmed that the chosen fmk pretreatment protocol inhibits the RSK CTK activity without significantly affecting MEK or ERK activity, we next focused on determining the effects of such fmk pretreatment on the CTK and N-terminal kinase (NTK) activities of endogenously expressed RSK in uninfected ARVMs, after α_1-adrenoceptor stimulation. As shown in Fig. 3A, α_1-adrenergic stimulation increased the phosphorylation of native RSK at Ser386, and this effect was significantly attenuated by fmk pretreatment, confirming inhibition of the RSK CTK activity. As illustrated in Fig. 1, the catalytic activity of the CTK domain determines the catalytic activity of the NTK domain (by regulating PDK1-mediated phosphorylation of the NTK activation loop), and the NTK domain is responsible for phosphorylating cellular RSK substrates, such as NHE1. To determine whether fmk inhibits NTK activation, we immunoprecipitated endog-

enous RSK from ARVMs after α_1-adrenergic stimulation and determined its ability to phosphorylate a recombinant fusion protein containing aa 625 to 815 of the NHE1 regulatory domain, in an in vitro kinase assay. As shown in Fig. 3B, when vehicle-treated ARVMs were exposed to phenylephrine, immunoprecipitated RSK exhibited a markedly increased ability to phosphorylate the NHE1 fusion protein in vitro, indicating increased NTK activity. However, when ARVMs were pretreated with fmk before exposure to phenylephrine, immunoprecipitated RSK exhibited a markedly reduced ability to phosphorylate the NHE1 fusion protein (Fig. 3B), indicating inhibition of α_1-adrenoceptor-mediated RSK NTK activation. It is noteworthy that the amount of RSK immunoprecipitated was unaltered by fmk pretreatment (Fig. 3B), supporting altered intrinsic NTK activity of immunoprecipitated RSK as the mechanism underlying the observed inhibition. To verify the mechanism of action of fmk, we next investigated its effect on RSK-mediated NHE1 phosphorylation when added to the in vitro kinase reaction, after ARVMs were exposed to phenylephrine and subsequent RSK immunoprecipitation. Once again, the amount of RSK immunoprecipitated was comparable among groups, but, under these conditions, fmk had no effect on the α_1-adrenoceptor-mediated increase in the in vitro phosphorylation of the NHE1 fusion protein (Fig. 3C). These data indicate that fmk has no direct inhibitory effect on the activated RSK NTK domain and support the concept that inhibition of CTK activity is the specific mechanism through which fmk inhibits NTK activation, in the relevant cellular context.

Having confirmed the efficacy and relative specificity of fmk as an inhibitor of RSK versus MEK or ERK activation in the cell type of interest, we next investigated whether fmk inhibits the phosphorylation of endogenous NHE1 in intact ARVMs. To achieve this, we used a method that we have recently described, which involves immunoprecipitation of NHE1 using a phospho-specific antibody that recognizes RSK-mediated phosphorylation sites in the regulatory domain of the exchanger (Snabaitis et al., 2006). Consistent with our previous data (Snabaitis et al., 2006), α_1-adrenergic stimulation produced a significant increase in endogenous NHE1 phosphorylation (Fig. 4A). It is noteworthy that this increase in NHE1 phosphorylation was attenuated by fmk pretreatment (Fig. 4A). We also carried out the same protocol in ARVMs infected with AdV-hNHE1, in an effort to boost the NHE1 signal through heterologous expression. After infection with AdV-NHE1, cells expressed HA-tagged proteins migrating at around 105 and 80 kDa (Fig. 4B), representing differentially glycosylated forms of NHE1 (Shrode et al., 1998). Heterologously expressed NHE-1, in particular the 80-kDa moiety, showed increased phosphorylation after α_1-adrenergic stimulation, and this response was again inhibited by fmk pretreatment (Fig. 4C). The observations that fmk, used under conditions that have been confirmed to inhibit RSK but not MEK or ERK (Figs. 2 and 3), inhibits phosphorylation of both the endogenous rat NHE1 and its heterologously expressed human ortholog provide strong evidence that RSK is the principal effector of NHE1 phosphorylation upon α_1-adrenoceptor-mediated activation of the MEK-ERK-RSK cascade in ARVMs.

Finally, we determined the effect of fmk on α_1-adrenoceptor-mediated stimulation of endogenous sarcolemmal NHE activity in ARVMs, which we have previously shown to be inhibited by upstream inhibition of the MEK-ERK-RSK cascade (Snabaitis et al., 2000). There was no significant differ-

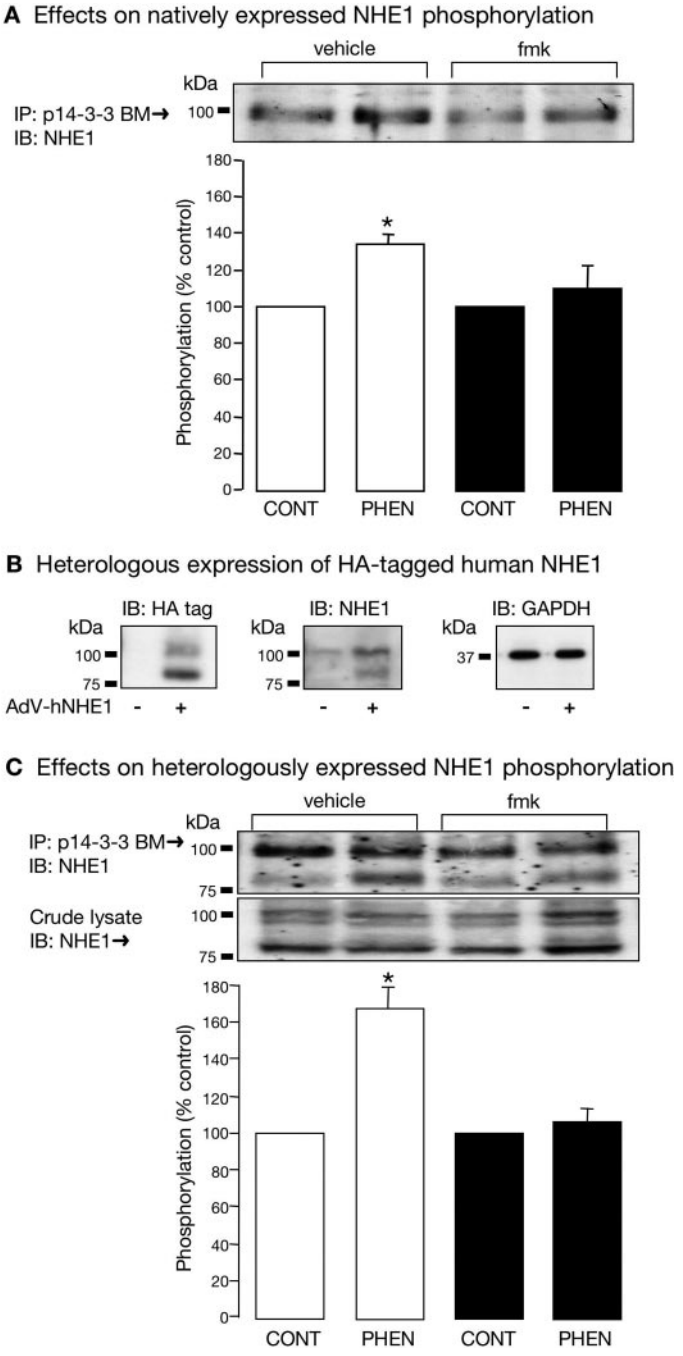

Fig. 4. Effects of fmk pretreatment on α_1-adrenoceptor-mediated phosphorylation of native or heterologously expressed NHE1 in ARVMs. A, phosphorylation of native NHE1 in uninfected ARVMs. B, heterologous expression of HA-tagged human NHE1 in ARVMs infected with AdV-hNHE1 (50 PFU/cell). C, phosphorylation of heterologously expressed HA-tagged human NHE1 in ARVMs infected with AdV-hNHE1 (50 PFU/cell). In A and C, phosphorylation was assessed by immunoprecipitation with a phospho-Ser 14-3-3 protein binding motif (p14-3-3BM) antibody followed by IB analysis using an NHE1 antibody. In B, NHE1 expression was detected by IB analysis using antibodies targeted at the HA tag or NHE1, as indicated. Cells were pretreated with 3 μM fmk or vehicle (0.03% DMSO) for 90 min before being exposed to PHEN at 10 μM for 3 min. The bar charts show quantitative data (mean ± S.E.M.) from five (A) or six (C) independent experiments. *, $P < 0.05$ versus appropriate CONT group.

ence between the study groups in basal pH_i, but the minimum pH_i that was achieved after washout of NH_4Cl (to induce intracellular acidosis) was lower in cells pretreated with fmk (Table 1). Figure 5 shows sarcolemmal NHE activity (as reflected by the H^+ J_H, under bicarbonate-free conditions) relative to pH_i, in the presence or absence of 10 μM phenylephrine, with or without fmk pretreatment. As expected (Yokoyama et al., 1998; Snabaitis et al., 2000), phenylephrine induced a rightward and upward shift of the J_H versus pH_i relationship, with significantly increased J_H values at pH_i 6.50 to 6.70 (Fig. 5). On its own, fmk pretreatment had no significant effect on pH_i-mediated regulation of sarcolemmal NHE activity, with a J_H versus pH_i relationship similar to that in controls pretreated with vehicle. Of particular note, however, is that pretreatment with fmk abolished the phenylephrine-induced increase in sarcolemmal NHE activity at pH_i 6.50 to 6.70. These data show that α_1-adrenoceptor-mediated stimulation of sarcolemmal NHE activity in the ARVM occurs through a mechanism that is sensitive to inhibition by fmk. The biochemical data presented above suggest that this fmk-sensitive mechanism is RSK-mediated phosphorylation of the regulatory domain of NHE1, the molecular homolog of the sarcolemmal NHE.

Discussion

We have taken advantage of the characterization of fmk as an effective and specific inhibitor of cellular RSK activation (Cohen et al., 2005; this study) to use this agent as a pharmacological tool to determine the role of RSK as a regulator of NHE1 phosphorylation and sarcolemmal NHE activity after α_1-adrenergic stimulation in adult myocardium. Our findings reveal, for the first time to our knowledge, that RSK is the principal regulator of NHE1 phosphorylation and sarcolemmal NHE activity after α_1-adrenergic stimulation in adult myocardium. The availability of fmk will now also allow determination of the role of RSK in NHE1 regulation by other (patho)physiologically relevant stimuli, such as angiotensin II, oxidative stress, and sustained intracellular acidosis, which have been shown to require activation of the MEK-ERK-RSK cascade for stimulation of sarcolemmal NHE activity (Gunasegaram et al., 1999; Snabaitis et al., 2002; Haworth et al., 2003).

Myocardial RSK activity is increased by ischemia and reperfusion (Takeishi et al., 1999), a setting in which NHE1 inhibition has been shown to afford significant cardioprotective benefit in numerous experimental studies (Avkiran, 2001; Avkiran and Marber, 2002). A study published during the preparation of the manuscript for this article reported intriguingly that transgenic expression of an RSK1 mutant, in which both the CTK and the NTK were rendered inactive by Lys/Ala substitutions in their ATP binding sites, enhanced the tolerance of mouse myocardium to ischemia and reperfusion-induced injury, probably through reduced sarcolemmal NHE activity (Maekawa et al., 2006). RSK activity is also significantly elevated in failing human myocardium (Takeishi et al., 2002), in which we have previously reported a significant increase in sarcolemmal NHE activity that arises from a post-translational mechanism (Yokoyama et al., 2000). In this context, we have recently demonstrated that increased myocardial RSK activity is recapitulated in a rabbit model of heart failure, in which NHE1 inhibition preserves myocardial morphology and function (Aker et al., 2004). It seems likely, therefore, that increased RSK activity may contribute to increased NHE1 activity and its detrimental consequences in myocardium in multiple pathological settings.

Despite their apparent therapeutic potential in experimental studies, clinical trials with direct NHE1 inhibitors (such as cariporide and eniporide) have yielded mixed results. These agents protect human myocardium against ischemia-

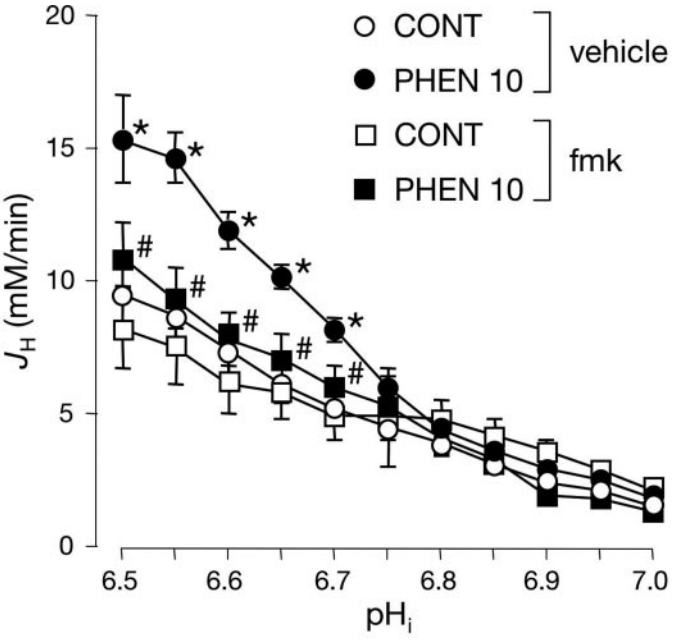

Fig. 5. Effects of fmk pretreatment on α_1-adrenoceptor-mediated stimulation of sarcolemmal NHE activity in ARVMs. H^+ J_H is shown at pH_i 6.50 to 7.00, determined during recovery from intracellular acidosis induced by transient exposure to 20 mM NH_4Cl (see *Materials and Methods*). Cells were pretreated with 3 μM fmk or vehicle (0.03% DMSO) for $\geq$90 min, before being exposed to PHEN at 10 μM for 3 min, concomitantly with NH_4Cl. Data are mean $\pm$ S.E.M., with n = 37 to 64 cells/group. *, $P < 0.05$ versus appropriate CONT group; #, $P < 0.05$ versus corresponding vehicle-treated group.

TABLE 1
Mean values for resting pH_i (measured before the NH_4Cl pulse) and minimum pH_i (measured immediately after NH_4Cl washout) in the four study groups
All values mean $\pm$ S.E.M. (n = 37–64 cells/group).

	Vehicle		fmk	
	Control	Phenylephrine	Control	Phenylephrine
Resting pH_i	7.21 $\pm$ 0.02	7.18 $\pm$ 0.02	7.20 $\pm$ 0.02	7.13 $\pm$ 0.02
Minimum pH_i	6.30 $\pm$ 0.04	6.36 $\pm$ 0.06	6.05 $\pm$ 0.06*	6.13 $\pm$ 0.04*

* $P < 0.05$ vs. corresponding vehicle-treated group.

and reperfusion-induced injury in certain settings, such as in high-risk patients undergoing coronary artery bypass graft surgery (Théroux et al., 2000; Boyce et al., 2003), where prerequisites for NHE1 inhibitor efficacy may be readily fulfilled (Avkiran and Marber, 2002). It is important to note, however, that in the recent EXPEDITION trial (the findings of which were presented at the American Heart Association meeting in November 2003; Mentzer, 2003), the significant cardioprotection afforded by cariporide treatment was tempered by serious noncardiac adverse effects (increased incidence of cerebrovascular events and mortality). Thus, the therapeutic application of agents that directly and globally inhibit the ubiquitously expressed NHE1 protein remains in abeyance, at least until the mechanisms of the adverse effects are delineated and the feasibility of dissociating these from the beneficial effects is ascertained. In the meantime, improved understanding of the molecular mechanisms that regulate the sarcolemmal NHE may allow alternative approaches to the therapeutic manipulation of exchanger activity to be developed. In this context, the present study suggests that RSK inhibition may attenuate phosphorylation-mediated enhancement of sarcolemmal NHE activity under conditions of marked intracellular acidosis, without affecting physiological regulation of exchanger activity under resting conditions (Fig. 5). The recent discovery of agents that specifically target RSK, such as fmk (Cohen et al., 2005) and SL0101, an inhibitor of RSK NTK activity that has been extracted from the tropical plant *Forsteronia refracta* (Smith et al., 2005), should help provide the pharmacological tools that are necessary to determine the potential of RSK as a therapeutic target, in clinically relevant models of cardiac disease.

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