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Novel Role for p90 Ribosomal S6 Kinase in the Regulation of Cardiac Myofilament Phosphorylation*[S]

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In myocardium, the 90-kDa ribosomal S6 kinase (RSK) is activated by diverse stimuli and regulates the sarcolemmal Na^+/H^+ exchanger through direct phosphorylation. Only limited information is available on other cardiac RSK substrates and functions. We evaluated cardiac myosin-binding protein C (cMyBP-C), a sarcomeric regulatory phosphoprotein, as a potential RSK substrate. In rat ventricular myocytes, RSK activation by endothelin 1 (ET1) increased cMyBP-C phosphorylation at Ser^{282}, which was inhibited by the selective RSK inhibitor D1870. Neither ET1 nor D1870 affected the phosphorylation status of Ser^{273} or Ser^{302}, cMyBP-C residues additionally targeted by cAMP-dependent protein kinase (PKA). Complementary genetic gain- and loss-of-function experiments, through the adenoviral expression of wild-type or kinase-inactive RSK isoforms, confirmed RSK-mediated phosphorylation of cMyBP-C at Ser^{282}. Kinase assays utilizing as substrate wild-type or mutated (S273A, S282A, S302A) recombinant cMyBP-C fragments revealed direct and selective Ser^{282} phosphorylation by RSK. Immunolabeling with a $Ser(P)^{282}$ antibody and confocal fluorescence microscopy showed RSK-mediated phosphorylation of cMyBP-C across the C-zones of sarcomeric A-bands. In chemically permeabilized mouse ventricular muscles, active RSK again induced selective Ser^{282} phosphorylation in cMyBP-C, accompanied by significant reduction in Ca^{2+} sensitivity of force development and significant acceleration of cross-bridge cycle kinetics, independently of troponin I phosphorylation at Ser^{22}/Ser^{23}. The magnitudes of these RSK-induced changes were comparable with those induced by PKA, which phosphorylated cMyBP-C additionally at Ser^{273} and Ser^{302}. We conclude that Ser^{282} in cMyBP-C is a novel cardiac RSK substrate and its selective phosphorylation appears to regulate cardiac myofilament function.

The 90-kDa ribosomal S6 kinase (p90 RSK or RSK),[3] also known as the mitogen-activated protein kinase-activated protein kinase-1 (MAPKAP-K1) family, comprises three principal isoforms: RSK1 (encoded by the *RPS6KA1* gene), RSK2 (*RPS6KA3*), and RSK3 (*RPS6KA2*) (1). An additional isoform, RSK4 (*RPS6KA6*), has also been identified, but evidence suggests that this isoform behaves in an atypical manner in terms of its expression and function (2). All four isoforms are characterized by the presence of two functionally distinct kinase domains, the N-terminal kinase (NTK) and the C-terminal kinase (CTK) (1). RSKs bind and are activated by extracellular signal-regulated kinases 1 and 2 (ERK1/2) through a sequential process that involves ERK-mediated phosphorylation of the CTK, CTK-mediated autophosphorylation of the RSK hydrophobic motif to create a docking site for 3′-phosphoinositide-dependent kinase 1 (PDK1), and PDK1-mediated phosphorylation of the NTK (1). Although the only known substrate of the CTK is the RSK hydrophobic motif, multiple cellular substrates are phosphorylated by the NTK, a serine/threonine kinase of the AGC kinase family (1). Consistent with this, RSK activity has been proposed to play important regulatory roles in diverse cellular processes, including cell survival, growth, proliferation, and migration (1). Pharmacological inhibition of the RSK NTK has been shown to inhibit the proliferation of cancer cell lines (3), and a recent study has shown that RSK activity is necessary to induce epithelial cell motility and invasiveness (4). These and other complementary observations suggest that RSK may represent a viable drug target in some carcinomas (5, 6).

With regard to cardiovascular biology, considerable data have accumulated to indicate that RSK phosphorylates the Na^+/H^+ exchanger (NHE) isoform 1 (NHE1) at Ser^{703} (7) and thereby mediates increased cardiac sarcolemmal NHE activity in response to diverse stress stimuli (8–11). In view of the proposed involvement of NHE1 activity in the pathogenesis of cardiac ischemic injury (12) and remodeling (13), such findings have led to the consideration of RSK as a potential therapeutic target for cardiac protection (14). Nevertheless, only limited information is available on other RSK substrates and functions in the heart, which may include roles in the phosphorylation of the inhibitory subunit of cardiac troponin I (cTnI) (15), the induction of prorenin-converting enzyme in ischemic and diabetic myocardium (16), and the prolongation

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Methods and additional references and a table.

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[3] The abbreviations used are: RSK, 90-kDa ribosomal S6 kinase; ARVM, adult rat ventricular myocytes; cMyBP-C, cardiac myosin-binding protein C; cTnI, cardiac troponin I; cTnI-Ala_2, cTnI in which Ser^{22}/Ser^{23} are substituted by nonphosphorylatable Ala; CTK, C-terminal kinase; ET1, endothelin 1; ki, kinase-inactive; NHE, Na^+/H^+ exchanger; NTK, N-terminal kinase; *p*Ca, -log[Ca^{2+}]; pCa_{50}, *p*Ca at 50% maximum force.

of cardiac repolarization in response to reactive oxygen species (17).

In the present study, we provide evidence from complementary genetic, biochemical, pharmacological, and physiological experiments for a novel cardiac RSK substrate, namely the sarcomeric protein cardiac myosin-binding protein C (cMyBP-C), through the phosphorylation of which RSK regulates myofilament function. Specifically, our data indicate that RSK phosphorylates cMyBP-C selectively at Ser^{282} (amino acid numbering refers to the mouse sequence) and that such phosphorylation is associated with reduced myofilament Ca^{2+} sensitivity and accelerated cross-bridge cycle kinetics in ventricular myocardium.

EXPERIMENTAL PROCEDURES

Expanded methodology is provided in the supplemental Methods. Previously published methods were used for key techniques such as the isolation and culture of ventricular myocytes from the adult rat heart (18), adenoviral vector construction and myocyte infection (11, 19), *in vitro* phosphorylation of recombinant and native proteins (20, 21), immunoblot analysis (20), immunolabeling and confocal microscopy (21), and assessment of myofilament function in skinned mouse ventricular trabeculae (21). Quantitative data are given as mean ± S.E. Statistical comparisons were by paired or unpaired Student's *t* test, as appropriate, when comparing data between two groups, or by analysis of variance (ANOVA) followed by the Bonferroni test, when comparing data between multiple groups. $p < 0.05$ was considered significant.

RESULTS

In a previous study on myofilament protein phosphorylation by protein kinase D (PKD) (19), we observed an increase in cMyBP-C phosphorylation at Ser^{282} in adult rat ventricular myocytes (ARVM) exposed to ET1, which occurred independently of cellular PKD activity. The amino acid sequence immediately N-terminal to Ser^{282} in cMyBP-C (AGRRT**S** in mouse and GGRRI**S** in human; bold indicates the phosphorylated Ser residue) conforms to one of the two motifs that are commonly targeted in RSK NTK substrates (RXRXX**S** or RRX**S**; bold indicates the phosphorylated Ser residue and underlined indicates the required Arg residues) (22, 23). Furthermore, in cardiac myocytes, ET1 is a potent activator of the MEK-ERK-RSK signaling pathway (18). These observations led us to explore the potential role of the MEK-ERK-RSK pathway in ET1-induced cMyBP-C phosphorylation at Ser^{282}, initially through a pharmacological approach. U0126, a selective inhibitor of MEK (24), inhibited ET1-induced cMyBP-C phosphorylation at Ser^{282} (Fig. 1*A*). As expected, U0126 also inhibited MEK-mediated phosphorylation of ERK at Thr^{202}/Tyr^{204}, ERK-mediated phosphorylation of the RSK CTK at Thr^{577}, and CTK-mediated autophosphorylation of the RSK linker region at Ser^{386} (Fig. 1*A*). To explore the role of the RSK NTK, relative to its upstream activator kinases MEK, ERK, and the RSK CTK, in ET1-induced cMyBP-C phosphorylation at Ser^{282}, we also investigated the effects of D1870, a recently characterized selective inhibitor of the RSK NTK in RSK1 and RSK2 isoforms (25). Like U0126, D1870 also inhibited ET1-induced cMyBP-C phosphorylation at Ser^{282} (Fig. 1*B*). However, unlike U0126, D1870 increased MEK-mediated phosphorylation of ERK, ERK-mediated phosphorylation of the RSK CTK, and CTK-mediated autophosphorylation of the RSK linker region (Fig. 1*B*), in the basal state and following ET1 stimulation, which is consistent with recent evidence that the RSK NTK participates in a negative feedback loop that regulates the MEK-ERK-RSK pathway (25, 26). The consistent effects of U0126 and D1870 on ET1-induced cMyBP-C phosphorylation at Ser^{282}, together with the contrasting effects of these inhibitors on the activation of upstream elements of the relevant pathway, suggest that Ser^{282} may be a novel substrate for the RSK NTK in cardiac myocytes, on the assumption that D1870 specifically inhibits the NTK of RSK isoforms that are expressed in these cells.

To investigate the effects of D1870 on the NTK activity of endogenous RSK isoforms in ARVM, we immunoprecipitated these isoforms using a pan-RSK antibody and carried out an *in vitro* kinase assay, utilizing as substrate a recombinant protein that contains the C-terminal regulatory domain of the Na^+/H^+ exchanger NHE1, which is an established substrate for the RSK NTK (11). D1870, when added to the assay mixture after immunoprecipitation, completely abolished NHE1 phosphorylation, reflecting potent inhibition of the NTK activity of immunoprecipitated RSK isoforms (Fig. 2*A*, *top panel*). Interestingly, pretreatment of ARVM with D1870 prior to ET1 stimulation and RSK immunoprecipitation also inhibited NHE1 phosphorylation, likely reflecting the high affinity of D1870 for the RSK NTK (Fig. 2*A*, *bottom panel*). These findings confirm that D1870 is a potent inhibitor of the NTK of endogenous RSK isoforms in ARVM.

We next explored whether RSK could directly phosphorylate cMyBP-C at Ser^{282}, in a manner that is inhibited by D1870. In an *in vitro* kinase assay, immunoprecipitated endogenous RSK isoforms (data not shown) and recombinant active RSK2 (Fig. 2*B*) catalyzed marked phosphorylation of Ser^{282} in a recombinant substrate protein comprising the c1c2 domain (amino acids 53–362) of human cMyBP-C. RSK2-mediated Ser^{282} phosphorylation was abolished by D1870 and inhibited to a lesser extent by H89 (Fig. 2*B*), which is a broad spectrum inhibitor that targets multiple kinases including RSK2 (27) but is commonly used to inhibit PKA. As expected, the PKA catalytic subunit also induced marked phosphorylation of Ser^{282} in the recombinant cMyBP-C c1c2 substrate; however, such phosphorylation was unaffected by D1870 but completely abolished by H89 (Fig. 2*B*). These findings indicate that the RSK NTK can directly phosphorylate cMyBP-C at Ser^{282} and that D1870 does not inhibit PKA. The selectivity of D1870 was further confirmed in intact ARVM, in which pretreatment with this inhibitor had no effect on the phosphorylation of cTnI at Ser^{22}/Ser^{23} (Fig. 2*C*, *left panel*) following β-adrenoreceptor stimulation by isoproterenol, a response that is mediated by PKA. D1870 also had no effect on ET1-induced phosphorylation of cTnI at the same sites (Fig. 2*C*, *right panel*), which occurs through the PKC-PKD signaling axis (19, 20). Taken together, our data indicate that D1870 is a potent and selective inhibitor of the RSK NTK *in vitro* and in

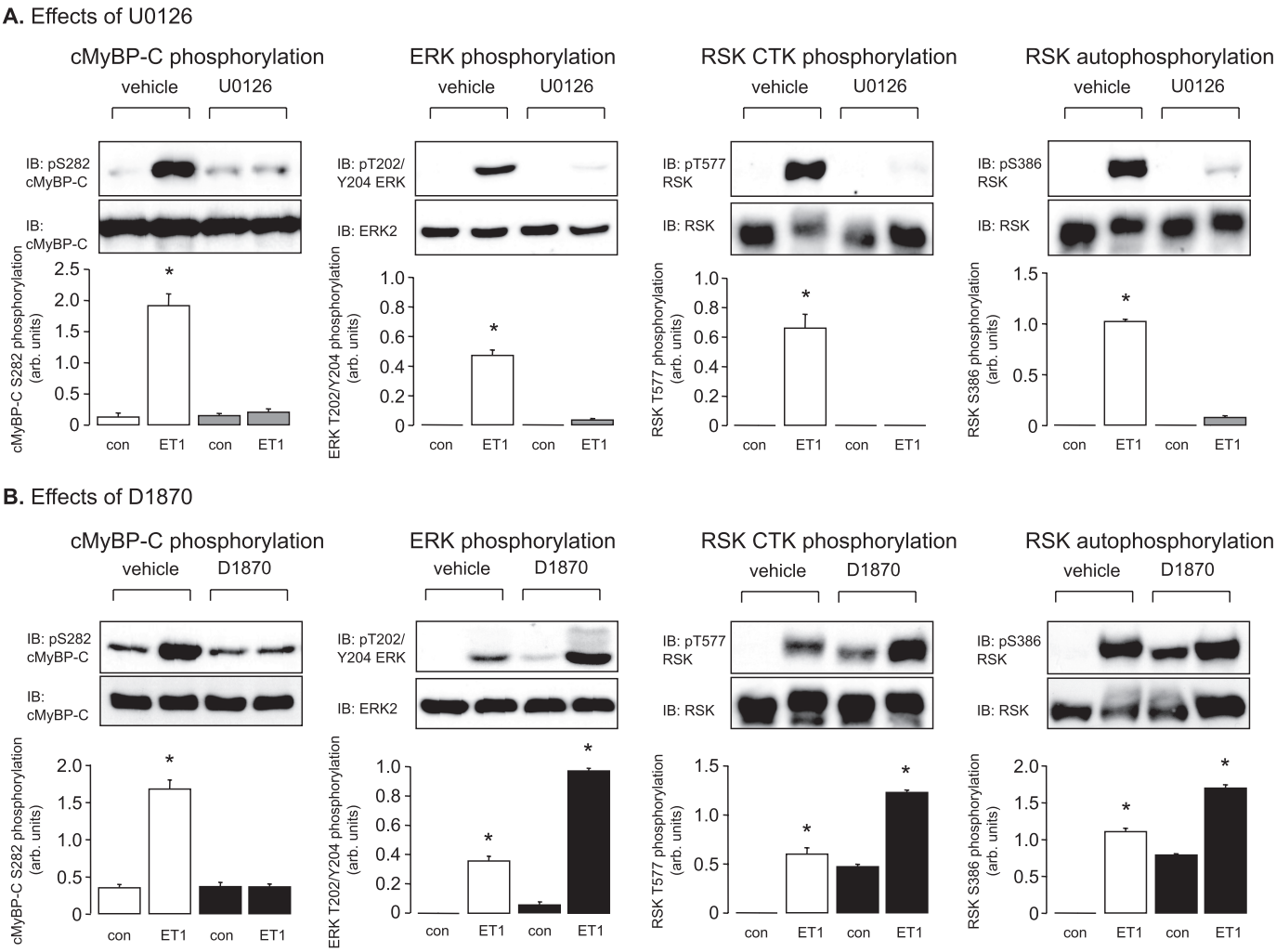

FIGURE 1. **Effects of the MEK inhibitor U0126 (*A*) or the RSK inhibitor D1870 (*B*) on ET1-induced phosphorylation of cMyBP-C at Ser^{282}, ERK at Thr^{202}/Tyr^{204}, RSK at Thr^{577}, or RSK at Ser^{386} in ARVM.** Cells were exposed to vehicle control or ET1 (50 nM for 10 min) following pretreatment with vehicle, U0126 (3 μM for 10 min) or D1870 (1 μM for 60 min). cMyBP-C, ERK, or RSK phosphorylation status was determined by immunoblot (*IB*) analysis using the indicated phosphospecific antibodies, with protein loading confirmed by immunoblot analysis using antibodies against total protein. Individual immunoblots illustrate representative experiments, and *bar charts* show quantitative data ($n = 6–8$). *, $p < 0.05$ *versus* corresponding control (*con*) group. *Error bars*, S.E.

intact ARVM and that its effects on cMyBP-C phosphorylation at Ser^{282} are likely to occur through this mechanism.

To investigate the role of individual RSK isoforms in cMyBP-C phosphorylation at Ser^{282}, we next employed a complementary genetic approach, through the use of adenoviral vectors to heterologously express wild-type (wt) or kinase-inactive (ki) forms of RSK1 (RSK1wt and RSK1ki) and RSK2 (RSK2wt and RSK2ki) in ARVM. In control cells (which were infected with adenoviral vectors encoding β-galactosidase or enhanced GFP), ET1 once again induced a significant increase in cMyBP-C phosphorylation at Ser^{282}, and this response was abolished by pretreatment of cells with D1870 (Fig. 3*A*). Relative to control cells, heterologous expression of RSK1wt or RSK2wt increased the basal phosphorylation of cMyBP-C at Ser^{282} and amplified the response to ET1, with D1870 exerting an inhibitory effect on both the basal and the ET1-induced phosphorylation (Fig. 3*A*). Importantly, heterologous expression of RSK1ki or RSK2ki at similar levels to their wt counterparts markedly attenuated the ET1-induced phosphorylation of cMyBP-C at Ser^{282}, through a dominant negative effect, and any residual ET1 response was abolished by D1870 (Fig. 3*A*). These data provide evidence that Ser^{282} in cMyBP-C is a novel substrate for both RSK1 and RSK2 isoforms in cardiac myocytes. Intriguingly, heterologous expression of RSK1wt or RSK2wt also led to increased phosphorylation of cTnI at Ser^{22}/Ser^{23}, particularly following ET1 stimulation, in a manner that was inhibited by pretreatment with D1870 (Fig. 3*B*). These findings are consistent with a previous report of increased cTnI phosphorylation at Ser^{22}/Ser^{23} in hearts of mice with cardiac-specific expression of an RSK1 transgene (15) and suggest that overexpression of RSK isoforms in myocardium may be associated with a loss of physiological substrate specificity. In this context, it is important to note that ARVM with endogenous levels of RSK expression (control cells in this particular set of experiments (Fig. 3*B*) and uninfected cells in the earlier experiments (Fig. 2*C*)) exhibited ET1-induced increases in cTnI phosphorylation at Ser^{22}/Ser^{23} that were unaffected by D1870, indicating that such increases occurred through an RSK-independent pathway, most likely by PKD (19).

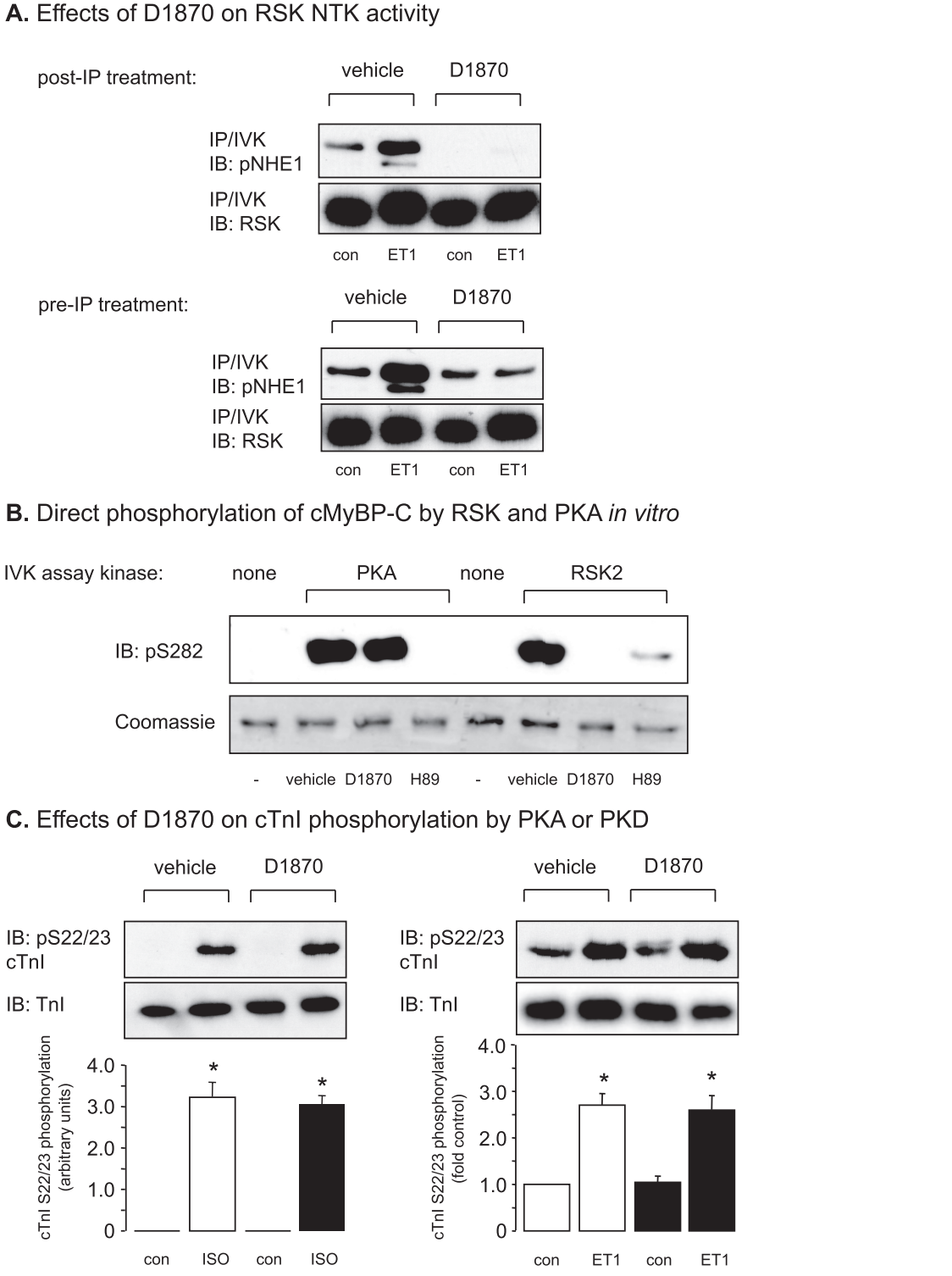

FIGURE 2. *A*, effects of D1870 on RSK NTK activity, as reflected by phosphorylation of a recombinant NHE1 fusion protein by endogenous RSK immunoprecipitated from ARVM, in an *in vitro* kinase (*IVK*) assay. Vehicle or D1870 (10 nM) was added to the reaction mixture after exposure of ARVM to ET1 (50 nM for 10 min) to activate RSK and subsequent RSK immunoprecipitation (*top panel*), or ARVM were pretreated with vehicle or D1870 (1 μM) for 60 min before exposure to ET1 to activate RSK and subsequent RSK immunoprecipitation (*IP*) (*bottom panel*). NHE1 phosphorylation status was determined by immunoblot (*IB*) analysis using a phosphospecific antibody and equal amounts of immunoprecipitated RSK in each sample confirmed by immunoblot analysis using an antibody against total RSK. Data are representative of three independent experiments. *B*, direct phosphorylation of cMyBP-C at Ser^{282} by RSK and PKA *in vitro*. A recombinant protein comprising the c1c2 fragment of human cMyBP-C was used as substrate in an *in vitro* kinase assay, in the presence or absence of D1870 (10 nM) or H89 (100 nM), and phosphorylation by the PKA catalytic subunit or RSK2 was detected by immunoblot analysis using Ser(P)^{282} phosphospecific cMyBP-C antibody. Equal protein loading was confirmed by Coomassie staining. *C*, effects of D1870 on PKA-mediated (*left panel*) or PKD-mediated (*right panel*) phosphorylation of cTnI at Ser^{22}/Ser^{23}, in ARVM exposed to isoproterenol (*ISO*; 10 nM for 10 min) or ET1 (50 nM for 10 min), respectively. cTnI phosphorylation status was determined by immunoblot analysis using Ser(P)^{22}/Ser(P)^{23} phosphospecific cTnI antibody, with protein loading confirmed by immunoblot analysis using an antibody against total cTnI. Individual immunoblots illustrate representative experiments, and *bar charts* show quantitative data ($n = 6$). *, $p < 0.05$ *versus* corresponding control (*con*) group. *Error bars*, S.E.

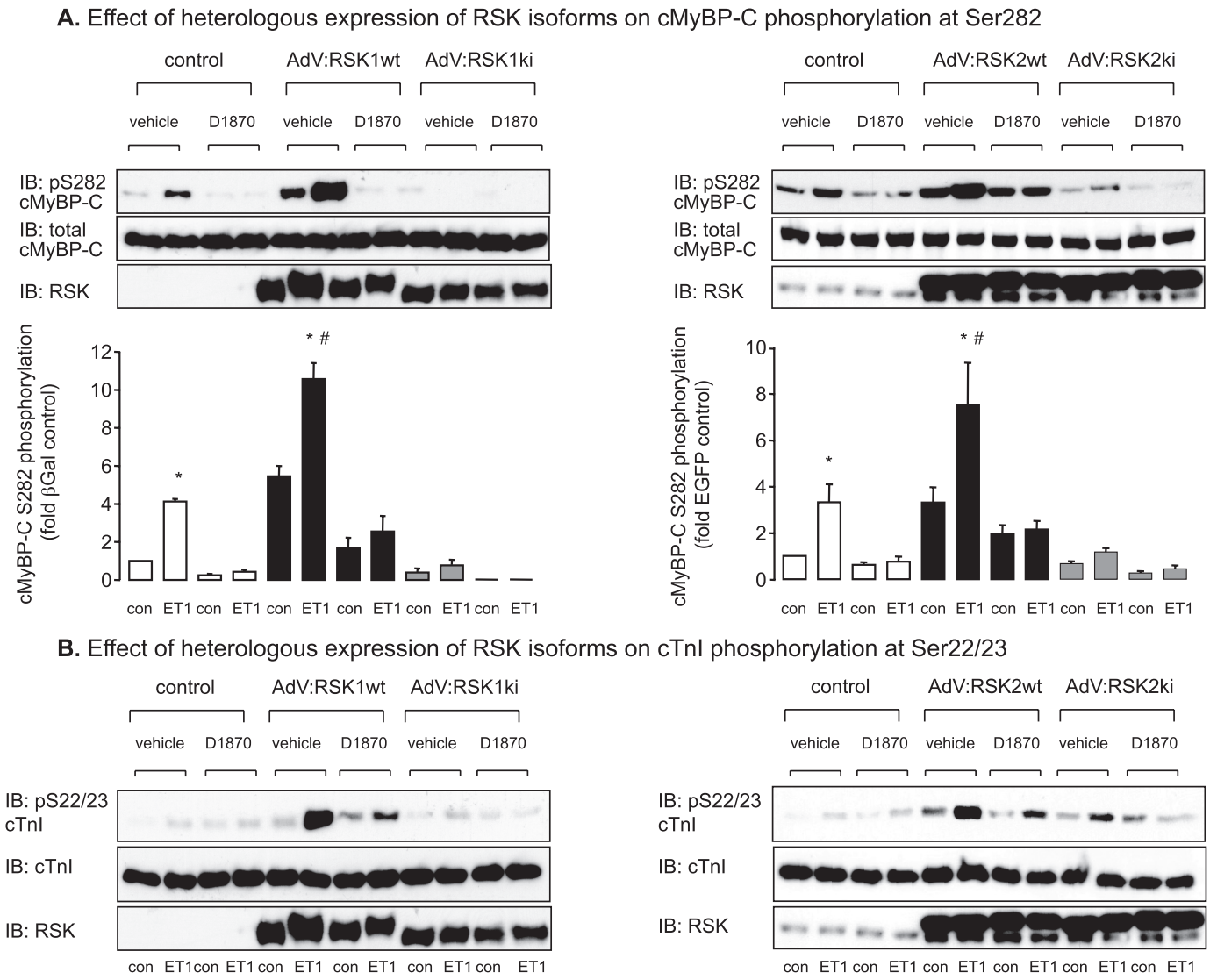

FIGURE 3. *A*, effects of heterologous expression of wild-type (wt) or kinase-inactive (ki) RSK isoforms on cMyBP-C phosphorylation at Ser^{282} in ARVM. Cells infected with AdV:βGal (control), AdV:RSK1wt or AdV:RSK1ki (*left panel*) or AdV:EGFP (control), AdV:RSK2wt or AdV:RSK2ki (*right panel*) were exposed to vehicle or ET1 (50 nM for 10 min) following pretreatment with vehicle or D1870 (1 μM for 60 min). cMyBP-C phosphorylation status was determined by immunoblot (*IB*) analysis using $Ser(P)^{282}$ phosphospecific cMyBP-C antibody, with protein loading confirmed by immunoblot analysis using an antibody against total cMyBP-C. Immunoblot analysis using a pan-RSK antibody confirmed comparable overexpression of wt and ki forms of RSK1 and RSK2. Individual immunoblots illustrate representative experiments, and *bar charts* show quantitative data on cMyBP-C phosphorylation at Ser^{282} ($n = 7$). *, $p < 0.05$ *versus* corresponding control (*con*) group; #, $p < 0.05$ *versus* corresponding ET1 group infected with AdV:βGal or AdV:EGFP. *Error bars*, S.E. *B*, effects of heterologous expression of wt or ki RSK isoforms on cTnI phosphorylation at Ser^{22}/Ser^{23} in ARVM. Experimental details are identical to those described in *A*, except that cTnI phosphorylation was determined by immunoblot analysis using a $Ser(P)^{22}/Ser(P)^{23}$ phosphospecific cTnI antibody, with protein loading confirmed by immunoblot analysis using an antibody against total cTnI. Data are representative of four independent experiments.

Although the experiments described above provide robust evidence that Ser^{282} in cMyBP-C is a novel RSK substrate, they do not exclude the possibility that other sites in cMyBP-C may also be RSK targets. To determine which of the phosphoacceptor sites in cMyBP-C that are established targets for PKA, namely Ser^{273}, Ser^{282}, and Ser^{302} (28), are also direct substrates for RSK, we carried out further *in vitro* kinase assays using recombinant active RSK2 or the PKA catalytic subunit and recombinant substrate proteins comprising the human cMyBP-C c1c2 domain, in wt form or mutated to singly replace each relevant Ser residue with nonphosphorylatable Ala (S273A, S282A, S302A). When *in vitro* phosphorylation was performed in the presence of [$Y^{32}P$]ATP and detected by autoradiography, wt c1c2 protein was phosphorylated by both kinases in a time-dependent manner (Fig. 4*A*). PKA-mediated phosphorylation was partially attenuated by each mutation, whereas RSK2-mediated phosphorylation was unaffected by the S273A and S302A mutations and completely abolished by the S282A mutation (Fig. 4*A*). These findings are consistent with the established ability of PKA to phosphorylate cMyBP-C directly at all three sites and additionally indicate that, in this system, RSK2 phosphorylates the c1c2 domain only at Ser^{282}. Indeed, at saturation, the stoichiometry of phosphorylation of wt c1c2 protein measured 1.3 ± 0.5 mol of phosphate/mol of substrate with RSK2 and 2.9 ± 0.4 mol of phosphate/mol of substrate with PKA ($n = 6$). Furthermore, when *in vitro* phosphorylation was performed in the presence of nonradiolabeled ATP and detected by immu-

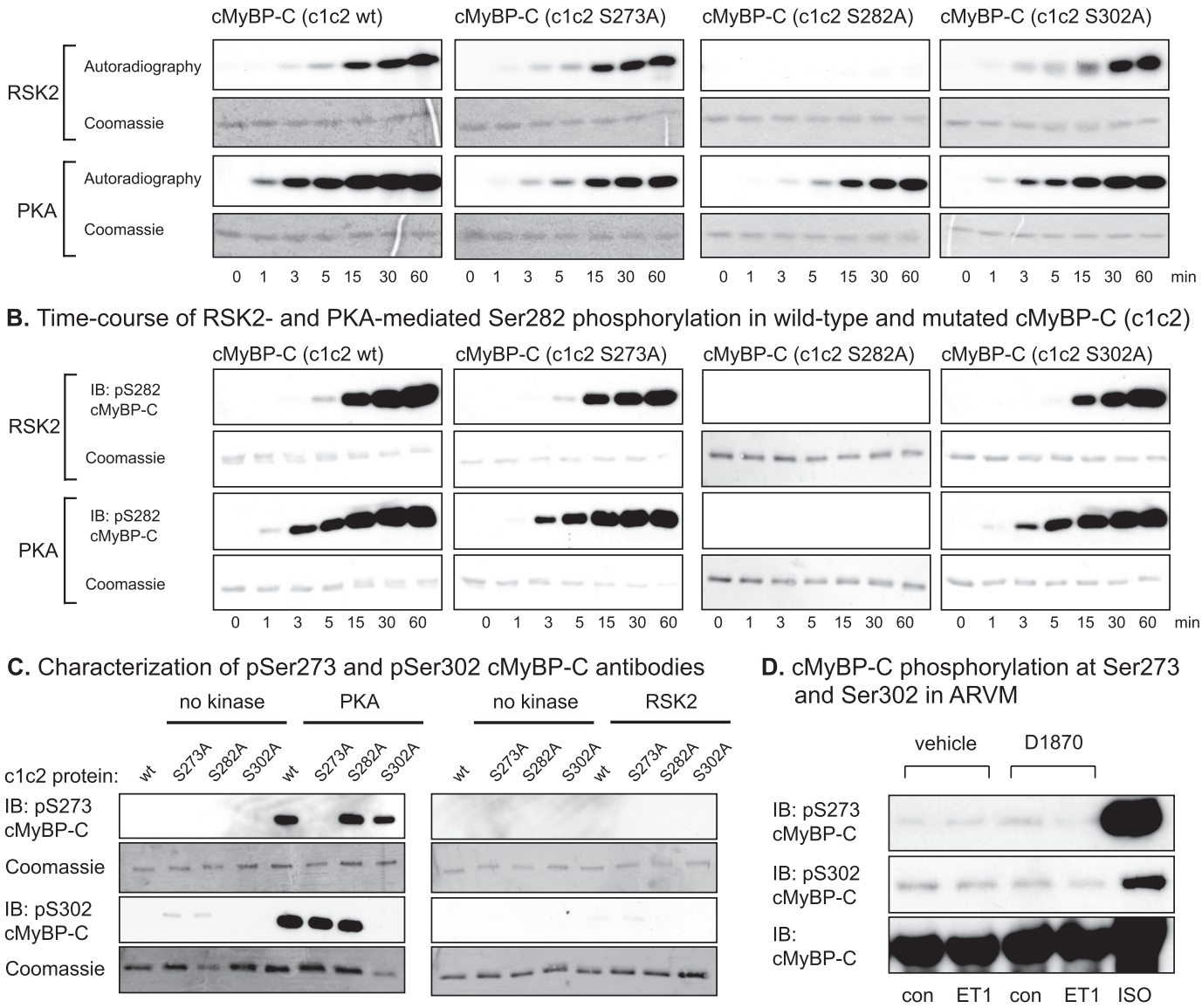

FIGURE 4. *A*, time course of RSK2- or PKA-mediated phosphorylation of wild-type (wt) and mutated cMyBP-C c1c2 domains. Recombinant proteins comprising the c1c2 fragment of human cMyBP-C, in wt form or carrying single Ser/Ala substitutions (S273A, S282A, or S302A), were used as substrate in an *in vitro* kinase (*IVK*) assay performed in the presence of [$Y^{32}P$]ATP, and phosphorylation by RSK2 or the PKA catalytic subunit was detected by autoradiography. *B*, time course of RSK2- or PKA-mediated Ser^{282} phosphorylation in wt and mutated cMyBP-C c1c2 domains. Experimental details are identical to those described in *A*, except that the *in vitro* kinase assay was performed in the presence of nonradiolabeled ATP, and phosphorylation by RSK2 or the PKA catalytic subunit was detected by immunoblot (*IB*) analysis using $Ser(P)^{282}$ phosphospecific cMyBP-C antibody. In both *A* and *B*, protein loading was confirmed by Coomassie staining, and the data are representative of three independent experiments. *C*, characterization of $Ser(P)^{273}$ and $Ser(P)^{302}$ phosphospecific cMyBP-C antibodies. Recombinant proteins comprising the c1c2 fragment of human cMyBP-C, in wt form or carrying single Ser/Ala substitutions (S273A, S282A, or S302A), were used as substrate in an *in vitro* kinase assay performed in the presence of nonradiolabeled ATP, and phosphorylation by the PKA catalytic subunit or RSK2 was detected by immunoblot analysis using $Ser(P)^{273}$ or $Ser(P)^{302}$ phosphospecific antibodies. Equal protein loading was confirmed by Coomassie staining. *D*, ET1-induced phosphorylation of cMyBP-C at Ser^{273} and Ser^{302} in ARVM. Cells were exposed to vehicle (*con*) or ET1 (50 nM for 10 min), following pretreatment with vehicle or D1870 (1 μM for 60 min). cMyBP-C phosphorylation status was determined by immunoblot analysis using $Ser(P)^{273}$ or $Ser(P)^{302}$ phosphospecific cMyBP-C antibody, with protein loading confirmed by immunoblot analysis using an antibody against total cMyBP-C. Cell extract from ARVM exposed to isoproterenol (*ISO*; 10 nM for 10 min) was used as a positive control. Data are representative of three independent experiments.

noblot analysis using the $Ser(P)^{282}$ phosphospecific antibody, no signal was detected following phosphorylation of S282A c1c2 protein by either kinase (Fig. 4*B*). In complementary work, we also used recently developed site-specific phospho-cMyBP-C antibodies (21, 29) to explore the potential phosphorylation of Ser^{273} and Ser^{302} by RSK *in vitro* and in intact ARVM. Immunoblot analysis of recombinant cMyBP-C c1c2 domain proteins phosphorylated *in vitro* by PKA or RSK2 confirmed the site specificity of these novel antibodies (following PKA-mediated phosphorylation) and the inability of RSK2 to phosphorylate Ser^{273} or Ser^{302} in this assay (Fig. 4*C*). Subsequent work with the $Ser(P)^{273}$ and $Ser(P)^{302}$ phosphospecific antibodies in ARVM revealed no effect of ET1 or D1870 on the phosphorylation status of Ser^{273} or Ser^{302},

whereas both were markedly enhanced in response to isoproterenol (Fig. 4*D*). Taken together, the above data indicate that RSK exclusively phosphorylates Ser^{282} in the unique N-terminal insert of cMyBP-C both *in vitro* and in intact cardiac myocytes and thus behaves in a distinct manner from PKA, which additionally phosphorylates Ser^{273} and Ser^{302}.

To explore the subcellular localization of cMyBP-C phosphorylation at Ser^{282} upon ET1-induced activation of endogenous RSK isoforms, we employed immunolabeling and confocal microscopy in conjunction with the Ser(P)^{282} phosphospecific antibody. Co-labeling of ARVM with antibodies that recognize cMyBP-C regardless of its phosphorylation status and α-actinin (an established Z-disc marker) revealed the expected decoration of the sarcomeres, with localization of cMyBP-C protein to the A-band, on either side of the central M-band between the Z-discs (Fig. 5*A*). Neither exposure to ET1 nor pretreatment with D1870 affected the sarcomeric localization of cMyBP-C (Fig. 5*A*). When similar co-labeling was performed using the Ser(P)^{282} phosphospecific antibody, no cMyBP-C signal was detectable in vehicle-treated ARVM, reflecting low basal phosphorylation (Fig. 5*B*). In ARVM exposed to ET1, however, the Ser(P)^{282} phosphospecific antibody decorated the sarcomeres with a pattern that was similar to that observed with the phosphorylation-insensitive cMyBP-C antibody, but at lower intensity (Fig. 5*B*). Importantly, such decoration of the sarcomeres with the Ser(P)^{282} phosphospecific cMyBP-C antibody after ET1 exposure was abolished by pretreatment of ARVM with D1870 (Fig. 5*B*). These findings indicate that, when activated, endogenous RSK isoforms in cardiac myocytes mediate cMyBP-C phosphorylation at Ser^{282} throughout the known subcellular localization of this sarcomeric protein.

Finally, to investigate the potential impact of RSK-mediated phosphorylation of cMyBP-C exclusively at Ser^{282} on cardiac myofilament function, we studied the effects of such phosphorylation on the Ca^{2+} sensitivity of force development and cross-bridge cycle kinetics in ventricular myocardium. Because RSK can also target cTnI at Ser^{22}/Ser^{23} *in vitro* (15) and when overexpressed in myocytes (Fig. 3*B*) (15), we used skinned myocardium from transgenic mice that only express cTnI in which Ser^{22}/Ser^{23} are substituted by nonphosphorylatable Ala (cTnI-Ala$_2$) (21, 30), to preclude functional effects arising from ancillary cTnI phosphorylation. In skinned myocytes from cTnI-Ala$_2$ hearts, RSK2 phosphorylated cMyBP-C selectively at Ser^{282}, whereas PKA additionally targeted Ser^{273} and Ser^{302} (Fig. 6*A*). As expected, no phosphorylation of cTnI at Ser^{22}/Ser^{23} could be detected following exposure to RSK2 or PKA (Fig. 6*B*) because the pertinent residues had been replaced by nonphosphorylatable Ala. In skinned ventricular trabeculae from cTnI-Ala$_2$ mice, RSK2-mediated phosphorylation significantly decreased the Ca^{2+} sensitivity of force development, as indicated by a rightward shift of the force-*p*Ca relationship (Fig. 7*A*, *left panel*) and a significant reduction in pCa_{50} (Fig. 7*B*). RSK2-mediated phosphorylation also accelerated cross-bridge cycle kinetics, as reflected by a significant increase in the rate of force redevelopment (k_{tr}) at submaximal force (Fig. 7*C*). Relative to RSK2, PKA-mediated phosphorylation had qualitatively and quantitatively similar effects

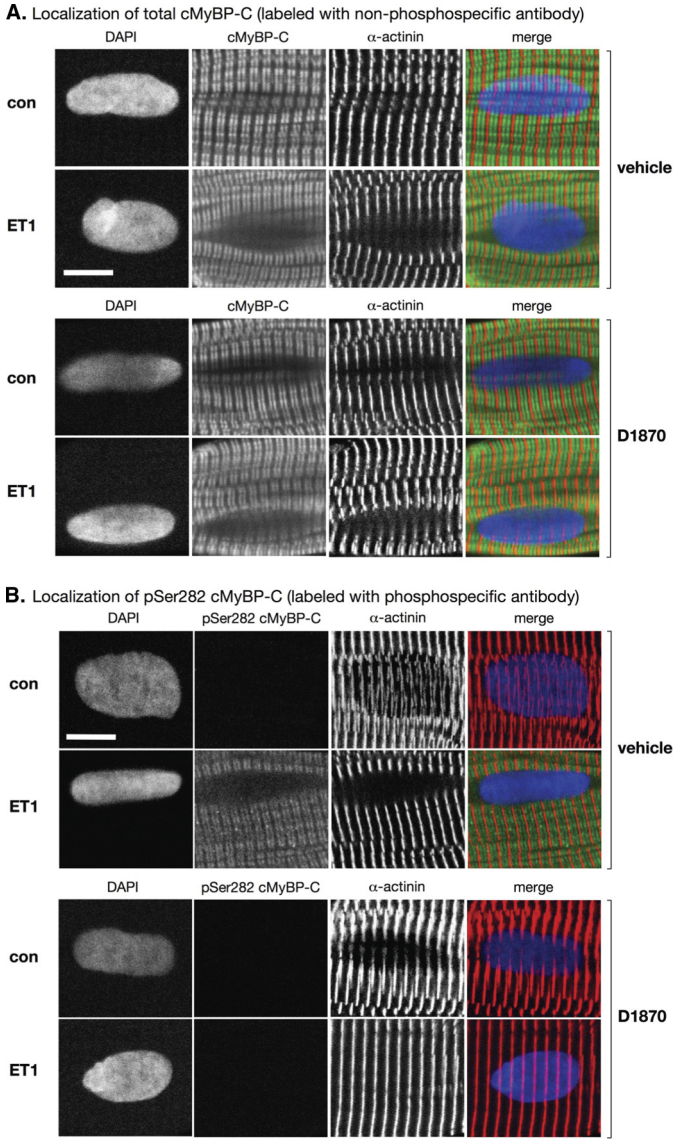

FIGURE 5. **Confocal microscope images showing the localization of total cMyBP-C (*A*) or cMyBP-C phosphorylated at Ser^{282} (*B*), in ARVM exposed to vehicle (*con*) or ET1 (50 nM for 10 min), following pretreatment with vehicle or D1870 (1 μM for 60 min).** The cells were additionally immunolabeled with an α-actinin antibody, to demarcate the Z-discs, and nuclei were stained with DAPI. The images were obtained from perinuclear regions of each cell. In the *merged* images, *red* indicates α-actinin labeling, *green* indicates total or Ser(P)^{282} cMyBP-C labeling, and the nuclei are stained *blue*. *Scale bars*, 10 μm.

on the Ca^{2+} sensitivity of force development (Fig. 7, *A*, *right panel*, and *B*) and cross-bridge cycle kinetics (Fig. 7*C*). In time-matched control experiments, no significant changes were observed in the Ca^{2+} sensitivity of force development or cross-bridge cycle kinetics in skinned trabeculae that were incubated for an identical time in the absence of either kinase (supplemental table). These findings indicate that RSK-mediated phosphorylation of Ser^{282} in cMyBP-C is paralleled by significant changes in key parameters of myofilament function, such as the Ca^{2+} sensitivity of force development and cross-bridge cycle kinetics, and that these changes are of comparable magnitude to those induced by PKA, which phosphorylates cMyBP-C additionally at Ser^{273} and Ser^{302}.

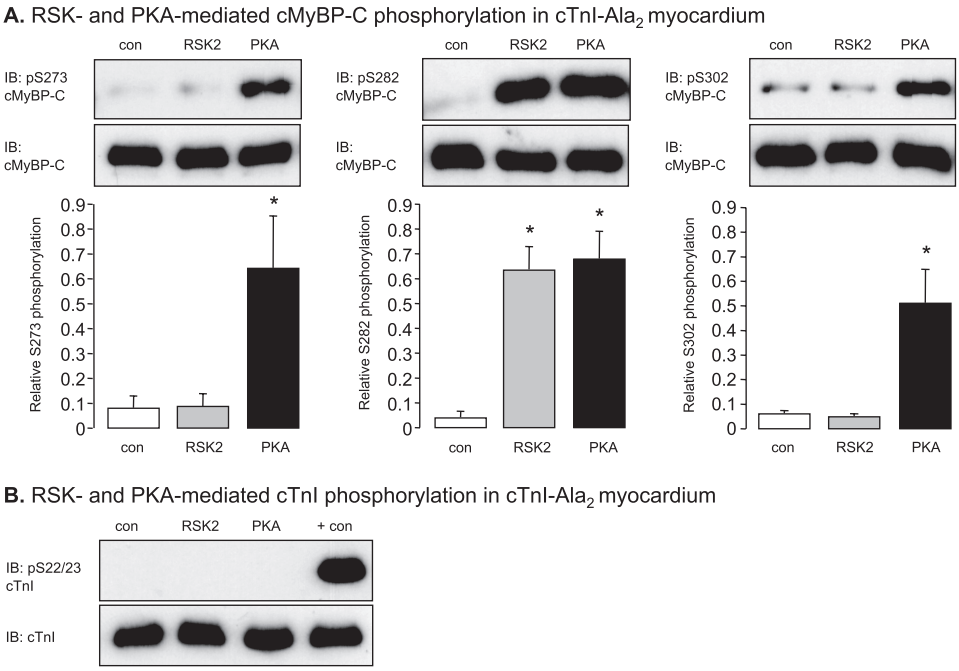

FIGURE 6. **RSK2- and PKA-mediated phosphorylation of cMyBP-C (*A*) and cTnI in skinned ventricular myocytes (*B*) from cTnI-Ala$_2$ mice, as detected by immunoblot (*IB*) analysis using (*A*) Ser(P)^{273}, Ser(P)^{282}, or Ser(P)^{302} phosphospecific cMyBP-C antibody and (*B*) Ser(P)^{22}/Ser(P)^{23} phosphospecific cTnI antibody.** Protein loading was confirmed by immunoblot analysis using total cMyBP-C or cTnI antibody, with positive control sample (+ *con*) obtained from ARVM exposed to isoproterenol (10 nM for 10 min). Individual immunoblots illustrate representative experiments, and *bar charts* show quantitative data on cMyBP-C phosphorylation ($n = 4$). *, $p < 0.05$ *versus* corresponding no-kinase control (*con*) group. *Error bars*, S.E.

DISCUSSION

Phosphorylation of sarcomeric proteins is a key mechanism through which cardiac myofilament function is regulated in response to neurohormonal stimuli (31). The present study provides novel evidence that (i) the sarcomeric protein cMyBP-C is a direct substrate for RSK; (ii) RSK-mediated phosphorylation of cMyBP-C occurs selectively at Ser^{282}; (iii) endogenous RSK isoforms in intact myocytes, upon their neurohormonal activation, mediate cMyBP-C phosphorylation at Ser^{282} across the expected A-band localization of this sarcomeric protein; and (iv) RSK-mediated phosphorylation of cardiac myofilaments reduces the Ca^{2+} sensitivity of force development and accelerates cross-bridge cycle kinetics independently of cTnI phosphorylation at Ser^{22}/Ser^{23}, most likely through phosphorylation of cMyBP-C at Ser^{282}. Previous studies on the regulation and roles of RSK isoforms in the cardiovascular system have revealed that cellular RSK activity is increased in response to multiple stimuli of relevance to cardiovascular physiology and pathophysiology, such as catecholamines (via α_1-adrenoceptors) (8, 11, 18), angiotensin II (32), ET1 (18), oxidative stress (33), intracellular acidosis (9), and ischemia/reperfusion (34). Furthermore, RSK expression and activity appear to be increased in animal models of heart failure and in failing human myocardium (35, 36). Although much attention to date has focused on RSK-mediated phosphorylation of NHE1 at Ser^{703} and the likely functional consequences of this regulatory pathway (10, 11, 14), novel cardiac substrates for RSK are beginning to emerge. Recent evidence has revealed that RSK phosphorylates Kv4.3, a component of the K^+ channel that carries the cardiac transient outward current (I_{to}), at Ser^{516} and Ser^{550}, and that this mechanism inhibits I_{to} and contributes to the prolongation of cardiac repolarization in response to oxidative stress (17). Our present data identify cMyBP-C as a novel substrate for RSK and provide evidence that RSK-mediated phosphorylation of cMyBP-C at Ser^{282} represents a hitherto unknown mechanism for regulating cardiac contractile function.

In recent years, a considerable amount of evidence has accumulated to suggest that the phosphorylation status of cMyBP-C is an important determinant of cardiac function in health and disease (see recent review by Barefield and Sadayappan in Ref. 37). Studies in genetically modified mice that express mutated cMyBP-C, in which Ser^{273}, Ser^{282}, and Ser^{302} (originally identified as phosphoacceptor residues targeted by PKA) (28) were concurrently replaced by nonphosphorylatable Ala (29, 38, 39) or phosphomimetic Asp (29, 40), indicate that phosphorylation of cMyBP-C is critically important in preserving sarcomeric integrity and myocardial contraction, in the basal state and following β-adrenergic stimulation or ischemia/reperfusion (29, 38–40). Importantly, some previous studies have also suggested a decrease in the global phosphorylation of cMyBP-C in human hearts with hypertrophic cardiomyopathy of varying genetic etiology (including several truncating mutations in the cMyBP-C gene *MYBPC3*) (41) and in the specific phosphorylation of Ser^{282} in myocardium of patients with end-stage heart failure and in canine myocardium from a model of pacing-induced heart failure (42). To the contrary, a recent report has shown that phosphorylation of cMyBP-C, globally or specifically at Ser^{282}, is not modified in myocardium from patients with hypertrophic cardiomyopathy associated with two distinct truncating mutations in *MYBPC3* (43). Phosphorylation of cMyBP-C was

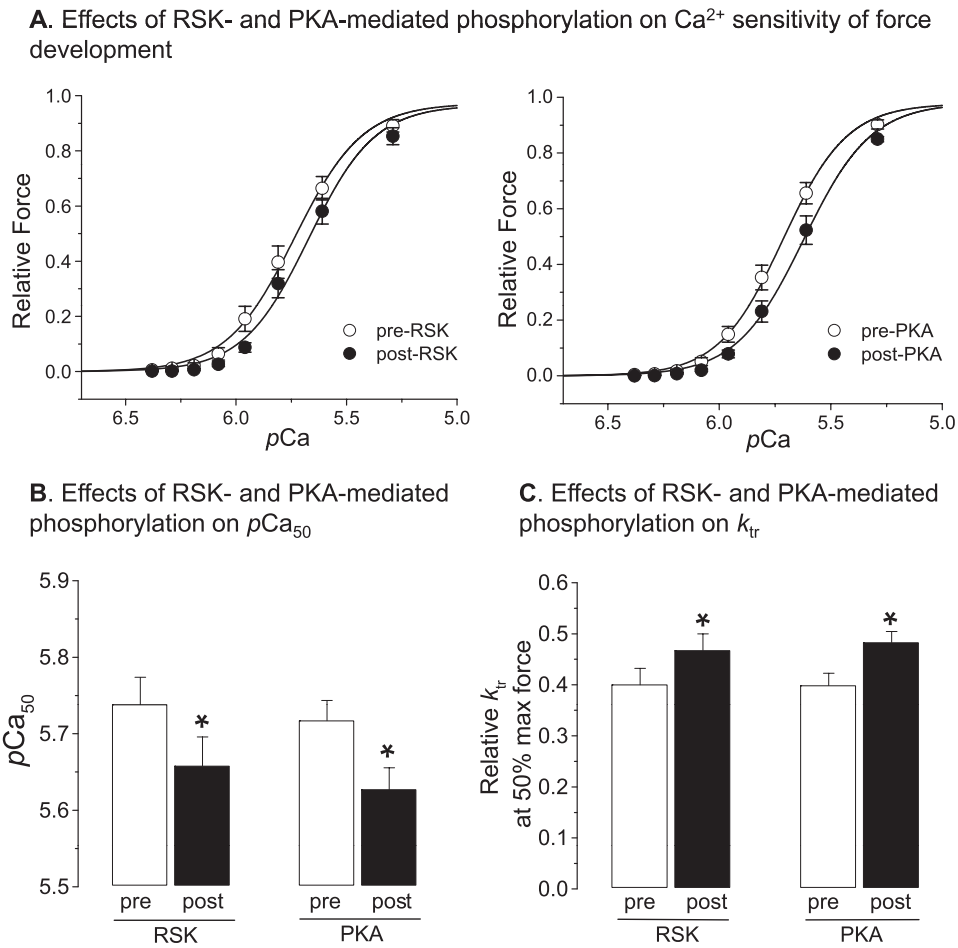

FIGURE 7. **Effects of RSK2- and PKA-mediated phosphorylation on (*A* and *B*) the Ca^{2+} sensitivity of force development and (*C*) cross-bridge cycle kinetics, in skinned ventricular trabeculae from cTnI-Ala_2 mice.** *A*, mean force-*p*Ca curves obtained before (*pre*; *open circles*) and after (*post*; *filled circles*) incubation of trabeculae with RSK2 (*left panel*) or PKA catalytic subunit (*right panel*). Force values were normalized to the maximum force, measured at *p*Ca 4.5. *B*, mean *p*Ca at 50% maximum force (pCa_{50}) obtained before (*pre*; *open bars*) and after (*post*; *filled bars*) incubation of trabeculae with RSK2 or PKA catalytic subunit. *C*, mean relative rate of force redevelopment (k_{tr}) at 50% maximum force obtained before (*pre*; *open bars*) and after (*post*; *filled bars*) incubation of trabeculae with RSK2 or PKA catalytic subunit. *, $p < 0.05$ *versus* corresponding pre-kinase value ($n = 7$; Student's paired *t* test). *Error bars*, S.E.

maintained in the presence of a significant reduction in the phosphorylation of cTnI at Ser^{22}/Ser^{23}, suggesting divergent regulation of signaling pathways that differentially regulate these functionally important sarcomeric phosphoproteins (43). Our data from the present study suggest that RSK provides an alternative to PKA for the phosphorylation of Ser^{282} in cMyBP-C, which may be of particular importance for the neurohormonal regulation of cMyBP-C phosphorylation under pathological conditions (such as heart failure) where the β-adrenoreceptor–PKA pathway is down-regulated (44). Indeed, under such conditions, RSK may act in concert with other kinases that are activated independently of the β-adrenoreceptor–PKA pathway (such as PKD, which we have shown recently to selectively target cMyBP-C at Ser^{302}) (21), to allow regulation of cMyBP-C phosphorylation at multiple sites. In this context, it is interesting to note that the expression of both RSK and PKD appears to be increased in various models of cardiac hypertrophy and failure (35, 36, 45) and in failing human myocardium (35, 46).

Although the functional importance of cMyBP-C phosphorylation is now firmly established (37), there is only limited information available on the potential roles of individual phosphorylation sites. This may be partly because PKA, the predominant kinase that mediates cMyBP-C phosphorylation under most physiological circumstances, targets all three of Ser^{273}, Ser^{282}, and Ser^{302} (28). Nevertheless, at least *in vitro*, Ser^{282} phosphorylation appears to play a unique role in facilitating PKA-mediated phosphorylation of Ser^{273} and Ser^{302}, potentially through a conformational change that renders the latter two sites more accessible (28). Importantly, emerging evidence from novel mouse models that express mutated cMyBP-C with selective replacement of Ser^{282} with nonphosphorylatable Ala or phosphomimetic Asp suggests that phosphorylation of this site is of functional importance also in the *in vivo* setting, in regulating both the phosphorylation of Ser^{273} and Ser^{302} and the contractile response following β-adrenergic stimulation (47). These observations raise the intriguing possibility that selective phosphorylation of Ser^{282} by RSK may prime the other sites for phosphorylation by distinct kinases, such as PKA, and thus have a broader functional impact. With respect to our present data on RSK-mediated regulation of myofilament function, it is interesting to note that, in our recent work in an identical preparation, selective phosphorylation of cMyBP-C at Ser^{302} by PKD was associated with

the acceleration of cross-bridge cycle kinetics without a change in myofilament Ca^{2+} sensitivity (21). In contrast, in the present study, selective phosphorylation of cMyBP-C at Ser^{282} by RSK was associated with both an acceleration of cross-bridge cycle kinetics and a reduction in myofilament Ca^{2+} sensitivity. These observations suggest that individual phosphorylation of Ser^{282} *versus* Ser^{302} may have differential impact on cardiac myofilament function.

As touched upon earlier, RSK activity has been implicated in the regulation of multiple cellular processes associated with tumor development and invasiveness, such that RSK inhibition has been proposed as a novel approach to pharmacotherapy in some carcinomas (4–6). Potential for cardiotoxicity continues to be an issue of significant clinical concern in the application of kinase inhibitors in cancer therapy (48), and the emerging cardiac roles of RSK should help inform the development of RSK inhibitors in this arena. As a corollary, our data also show that RSK inhibition in cardiac myocytes leads to enhanced activity of upstream components of the MEK-ERK-RSK signaling pathway. This observation is consistent with evidence from other cell types that the RSK NTK participates in a negative feedback loop that regulates the MEK-ERK-RSK pathway (25, 26). This has implications regarding the impact of increased ERK activity following RSK inhibition on both cancer and cardiac cell biology.

To conclude, the present study has revealed a novel substrate and role for RSK in the regulation of cardiac contractile function. Further work is now required to identify the individual role of RSK activity and to decipher the integrated actions of multiple convergent kinase pathways in the regulation of cMyBP-C phosphorylation and cardiac function *in vivo*, in physiological and pathological settings.

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