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# Phosphorylation of protein kinase A (PKA) regulatory subunit RI $\alpha$ by protein kinase G (PKG) primes PKA for catalytic activity in cells

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cAMP-dependent protein kinase (PKAc) is a pivotal signaling protein in eukaryotic cells. PKAc has two well-characterized regulatory subunit proteins, RI and RII (each having  $\alpha$  and  $\beta$  isoforms), which keep the PKAc catalytic subunit in a catalytically inactive state until activation by cAMP. Previous reports showed that the RI $\alpha$  regulatory subunit is phosphorylated by cGMP-dependent protein kinase (PKG) *in vitro*, whereupon phosphorylated RI $\alpha$  no longer inhibits PKAc at normal (1:1) stoichiometric ratios. However, the significance of this phosphorylation as a mechanism for activating type I PKA holoenzymes has not been fully explored, especially in cellular systems. In this study, we further examined the potential of RI $\alpha$  phosphorylation to regulate physiologically relevant “desensitization” of PKAc activity. First, the serine 101 site of RI $\alpha$  was validated as a target of PKGI $\alpha$  phosphorylation both *in vitro* and in cells. Analysis of a phosphomimetic substitution in RI $\alpha$  (S101E) showed that modification of this site increases PKAc activity *in vitro* and in cells, even without cAMP stimulation. Numerous techniques were used to show that although Ser<sup>101</sup> variants of RI $\alpha$  can bind PKAc, the modified linker region of the S101E mutant has a significantly reduced affinity for the PKAc active site. These findings suggest that RI $\alpha$  phosphorylation may be a novel mechanism to circumvent the requirement of cAMP stimulus to activate type I PKA in cells. We have thus proposed a model to explain how PKG phosphorylation of RI $\alpha$  creates a “sensitized intermediate” state that is in effect primed to trigger PKAc activity.

cAMP-dependent protein kinase (PKAc; with  $\alpha$ ,  $\beta$ , and  $\gamma$  isoforms)<sup>2</sup> is a pivotal cell signaling protein in eukaryotes (1–4).

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<sup>2</sup> The abbreviations used are: PKAc, protein kinase A catalytic subunit; PKG, protein kinase G; IS, inhibitor sequence; bRI $\alpha$ , recombinant bovine

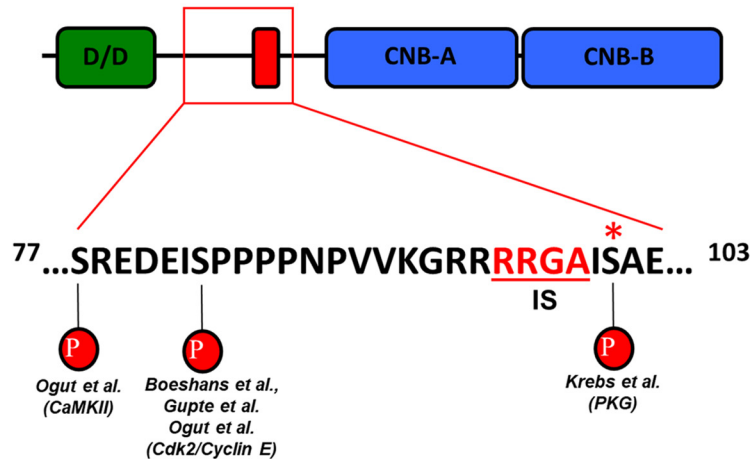
The catalytic activity of PKAc is in part controlled by four functionally non-redundant regulatory subunit proteins (RI $\alpha$ , RI $\beta$ , RII $\alpha$ , and RII $\beta$ ) (5–8), which bind PKAc in tetrameric “holoenzyme” complexes to maintain kinase inactivity until stimulation with cAMP (1, 9). The structural assembly of PKA holoenzymes and allosteric activation mechanism triggered by cAMP binding to R-subunits is well-understood (9–15). However, unique biochemical features of RI $\alpha$  may serve to activate PKAc via non-canonical, cAMP-independent mechanisms. The linker–hinge region of PKA regulatory subunit proteins contains the autoinhibitory motif that allows for selective inhibition of PKAc at the kinase active site; however, this inhibitor sequence (IS) differs significantly between RI and RII (11, 16). Although RII subunits have a PKA consensus phosphorylation sequence (RRXS) that is phosphorylated by PKAc, RI subunits have a “pseudosubstrate” IS (RRX(A/G)) that is unable to be phosphorylated by PKAc; therefore, substrate competition is required to trigger full PKA holoenzyme dissociation (17). Additionally, RI $\alpha$  subunit has three serine residues in the linker region that are putative phosphorylation sites: Ser<sup>77</sup>, Ser<sup>83</sup>, and Ser<sup>101</sup> (Fig. 1A) (18–20). Previously, the Ser<sup>101</sup> residue (P + 2 to the pseudosubstrate IS) was shown to be an *in vitro* substrate of cGMP-dependent protein kinase (PKG) (21, 22). Given the nature of the *in vitro* methodology used previously to assess phosphorylation of this site, as well as the lack of identity of a well-defined PKG consensus phosphorylation sequence, the physiological relevance of this putative phosphorylation site remains uncharacterized. To expand upon this work, experiments were aimed at validating *in vitro* studies with purified recombinant proteins and determining whether this unique mechanism of PKA activation happens in cell culture models.

Recent structural information acquired in the last 10–15 years concerning the nature of binding between PKAc and RI $\alpha$  allows for some conjecture about how modification of Ser<sup>101</sup> might lead to changes in type I PKA activity. The protein structure of the RI $\alpha$ –PKAc heterodimer was solved by X-ray crystallography techniques by Kim *et al.* (12), and this structure

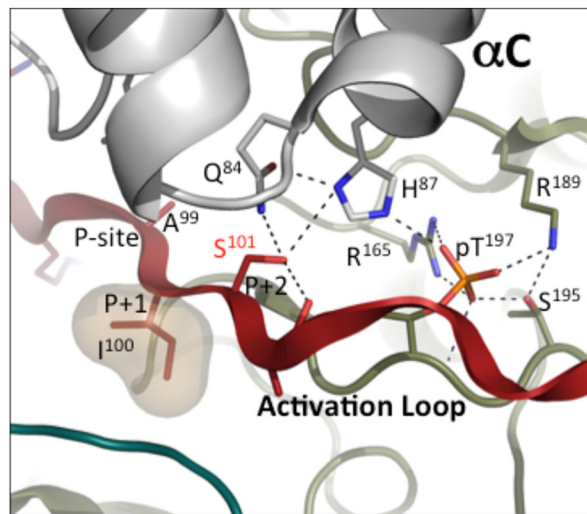
PKA regulatory subunit RI $\alpha$ ; MEF, mouse embryonic fibroblast; PCA, protein-fragment complementation assay; RLuc, *Renilla* luciferase; LiReC, ligand-regulated competition; HA, hemagglutinin; CNB, cyclic nucleotide binding; Fsk, forskolin; IBMX, isobutylmethylxanthine; co-IP, co-immunoprecipitation.

## R1a Ser<sup>101</sup> phosphorylation by PKG activates PKAc in cells

### A R1a: Domain Layout



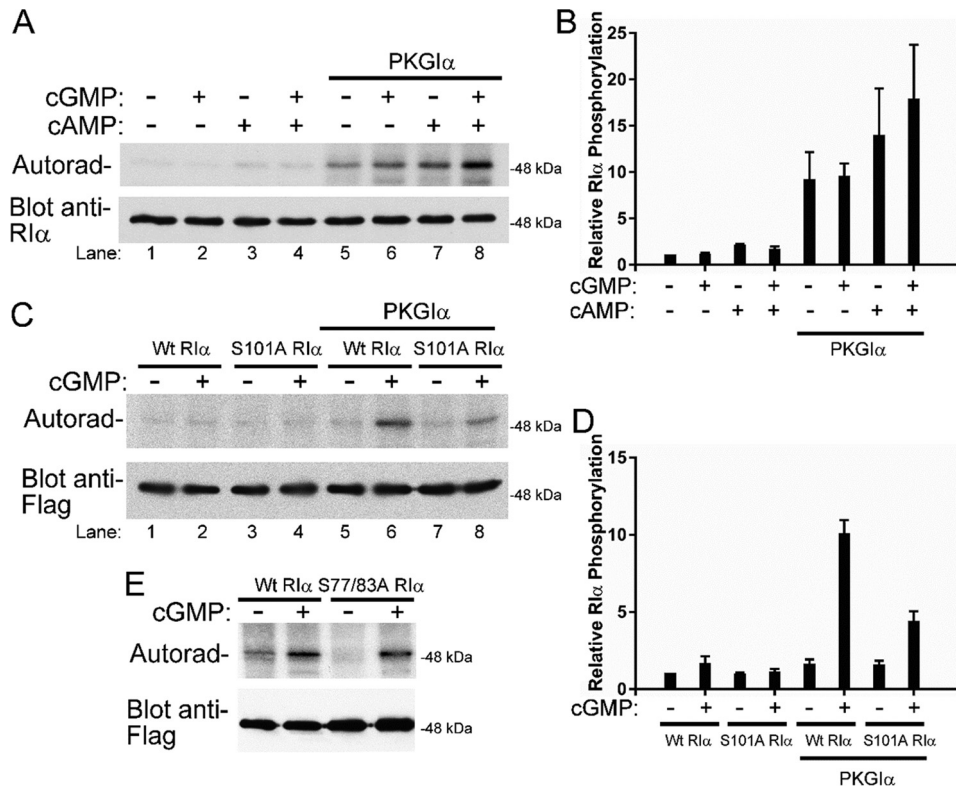
### B



**Figure 1.** *A*, cartoon diagram of R1 $\alpha$  domain layout, with emphasis on the linker region sequence and phosphorylation sites Ser<sup>77</sup>, Ser<sup>83</sup>, and Ser<sup>101</sup>. R1 $\alpha$  domains from the N terminus to the C terminus were as follows: dimerization/docking (D/D) domain, the pseudosubstrate inhibitory site (IS; sequence is shown in *red underlined text*), and tandem CNB domains. The presumed kinases for each putative phosphorylation site are listed below. The PKG phosphorylation site Ser<sup>101</sup> is indicated by a *red asterisk*. *B*, PyMOL structural representation of the R1 $\alpha$ -PKAc heterodimer complex (Protein Data Bank code 2qcs), focusing on the binding interface between the active site of PKAc and the linker region IS of R1 $\alpha$  (*light-gray cartoon*, PKAc N-lobe; *olive-green surface*, PKAc C-lobe; *teal cartoon*, R1 $\alpha$  CNB domains; *red cartoon with sticks*, R1 $\alpha$  linker region containing the IS to PKAc). The serine residue of interest in R1 $\alpha$  (Ser<sup>101</sup>, *red*) is depicted along with other critical residues at this binding interface, including 1) the pseudosubstrate alanine (Ala<sup>99</sup>) and *p* + 1 residue (Ile<sup>100</sup>) from the R1 $\alpha$  linker region; 2) two residues from the  $\alpha$ C-helix of PKAc (Gln<sup>84</sup> and His<sup>87</sup>) that form direct hydrogen bonds with the hydroxyl group of serine 101; and 3) activation loop phosphorylation site Thr(P)<sup>197</sup> and its neighboring residues in PKAc (Arg<sup>165</sup>, Arg<sup>189</sup>, and Ser<sup>195</sup>). Hydrogen bonds are depicted as *dashed lines*.

showed how the linker region of R1 $\alpha$  binds the active site cleft of PKAc. The IS makes direct contacts with residues from both the N-lobe and C-lobe of PKAc, whereas the CNB-A domain binds distally to the C-lobe. Because of the pseudosubstrate nature of the IS in R1 $\alpha$ , the high affinity binding of this motif to PKAc (in complex with ATP and two magnesium ions, Mg<sub>2</sub>ATP) presents a kinetic barrier for activation, whereas this is not critical for RII subunits capable of phosphorylation by PKAc (16, 23). The binding affinity of R1 $\alpha$  and PKAc in the presence of Mg<sub>2</sub>ATP is 0.1 nM *versus* 200 nM in the absence of nucleotide (24). ATP also binds with an affinity of 60 nM in the R1 $\alpha$  holoenzyme, whereas the *K<sub>m</sub>/K<sub>d</sub>* is 25  $\mu$ M for the free protein. This gives a rationale for why modification of Ser<sup>101</sup> could possibly perturb the binding interaction of the linker region of R1 $\alpha$  to PKAc.

A close-up view of this interfacial region helps to illustrate the importance of this residue in maintaining proper binding interactions with PKAc (Fig. 1B). Upon analysis of polar interactions in this region, one can see that the hydroxyl moiety of Ser<sup>101</sup> makes hydrogen bonds with residues from the  $\alpha$ C-helix of PKAc (particularly residues Gln<sup>84</sup> and His<sup>87</sup>). It is inferred that introduction of a phosphate group at this position will introduce steric hindrance that will likely alter the interaction of these residues. Furthermore, the interaction of Ser<sup>101</sup> with Gln<sup>84</sup> and His<sup>87</sup> facilitates a hydrogen-bond network near the activation-loop phosphorylation site Thr<sup>197</sup> in PKAc. Thus, we can further postulate that phosphorylation of Ser<sup>101</sup> will also bring about a negative charge-charge repulsion effect caused by the juxtaposition of two phosphate groups within this binding interface. These effects in combination would lend to open-



**Figure 2. PKGI $\alpha$  phosphorylates Rl $\alpha$  *in vitro* and in cells.** *A*, the upper panel shows autoradiography of *in vitro* PKGI $\alpha$  phosphorylation reactions conducted with purified recombinant bovine Rl $\alpha$  in complex with PKAc (holoenzyme). For all reactions, 0.6  $\mu$ g of holoenzyme was incubated with or without purified PKGI $\alpha$  in the presence of 1  $\mu$ M of the indicated cyclic nucleotides. The lower panel shows equal loading via an anti-Rl $\alpha$  immunoblot. *B*, densitometric quantification of three experiments performed as described in *A*, expressed as means  $\pm$  S.D. Statistical significance is indicated by planned comparisons. *C*, autoradiography and immunoblot analysis of phosphorylation reactions performed in HEK293T cells overexpressing FLAG-tagged versions of either wildtype or S101A mutant Rl $\alpha$  protein, with and without the presence of PKGI $\alpha$  and/or 2 h of cGMP stimulus (upper panel). The lower panel shows equal expression and isolation of FLAG-tagged Rl $\alpha$  proteins via an anti-FLAG immunoblot. *D*, densitometric quantification of three experiments performed as described in *C*, expressed as means  $\pm$  S.D. Statistical significance is indicated by planned comparisons. *E*, the upper panel shows autoradiography of phosphorylation reactions performed in HEK293T cells overexpressing FLAG-tagged constructs of either WT or serine 77 and serine 83 mutant versions of Rl $\alpha$  (S77/83A). All reactions were performed with overexpressed PKGI $\alpha$  with or without 2 h of 8-CPT-cGMP stimulus. The lower panel is an immunoblot using anti-FLAG antibodies as a loading control.

ing of the active site cleft of PKAc. Taking these observations under consideration, we hypothesized that phosphorylation of Ser<sup>101</sup> by PKG would promote enhanced PKAc activity by removing the additional requirement of substrate competition in Rl $\alpha$  holoenzyme activation. Finding evidence of direct PKG–PKA cross-talk in the context of cellular signaling would aid our understanding of how these two related pathways could regulate physiologically relevant systems where both proteins are expressed (*i.e.* smooth muscle and cardiac tissues) (25, 26).

**Results**

**PKGI $\alpha$  phosphorylates Rl $\alpha$  at serine 101 both *in vitro* and in cells**

To extend previous reports demonstrating that PKA Rl $\alpha$  could be phosphorylated by PKGI $\alpha$ , we performed *in vitro* phosphorylation reactions using purified recombinant bovine Rl $\alpha$  (bRl $\alpha$ )–PKAc holoenzyme and PKGI $\alpha$ . The reactions contained [ $\gamma$ -<sup>32</sup>P]ATP, and phosphate incorporation was determined by autoradiography. We observed robust phosphorylation of bRl $\alpha$  only in the presence of PKGI $\alpha$  (Fig. 2*A*, compare lanes 5–8 with lanes 1–4; quantification of data in Fig. 2*B*). Rl $\alpha$  was phosphorylated by PKGI $\alpha$  to a similar extent in the absence

and presence of cyclic nucleotides (lanes 5–8). These results are a bit unexpected, because cyclic nucleotide free and cAMP-bound PKGI $\alpha$  should not be as active as the cGMP-bound kinase. However, under the conditions used, it is likely that PKGI $\alpha$  activity was driven by PKGI $\alpha$  autophosphorylation and was cyclic nucleotide-independent. We originally reasoned that cAMP would disassociate bRl $\alpha$  from PKAc and thus provide easier access for PKGI $\alpha$  to phosphorylate Ser<sup>101</sup>; however, although there was a trend toward increased Rl $\alpha$  phosphorylation in the presence of cAMP, the difference did not reach significance (compare lane 5 with 7 and 6 with 8). Thus, it appears that holoenzyme association/dissociation has no effect on PKGI $\alpha$ 's access to the phosphorylation site.

Next, we examined whether PKGI $\alpha$  could phosphorylate Rl $\alpha$  in intact cells. HEK293T cells were transfected with expression vectors for PKGI $\alpha$  and FLAG-tagged WT or S101A-mutant human Rl $\alpha$ . The cells were labeled with [<sup>32</sup>P]orthophosphate, and the differences between WT and S101A-mutant Rl $\alpha$  phosphorylation were compared in the presence or absence of PKGI $\alpha$ , as well as with and without 2-h stimulus with 8-CPT-cGMP (a membrane-permeable analog of cGMP). In cells transfected with WT Rl $\alpha$  alone, we observed a small amount of basal Rl $\alpha$  phosphorylation, which was slightly increased by

## R1a Ser<sup>101</sup> phosphorylation by PKG activates PKAc in cells

treatment with 8-CPT-cGMP (Fig. 2C, compare lanes 1 and 2; quantification of data in Fig. 2D). In cells co-transfected with PKGI $\alpha$ , treatment with 8-CPT-cGMP induced a much higher level of RI $\alpha$  phosphorylation, which was dramatically reduced in cells transfected with PKGI $\alpha$  and S101A-mutant RI $\alpha$  (Fig. 2C, compare lane 2 with lane 6 and lane 6 with lane 8). The lower panel of Fig. 2C is an immunoblot using anti-FLAG antibodies, showing an equal expression of transfected FLAG-tagged RI $\alpha$  in all samples tested. Our results indicate that RI $\alpha$  Ser<sup>101</sup> is a specific phosphorylation target of PKGI $\alpha$  in cells.

In these cell-based experiments, the faint phosphorylation signal observed for RI $\alpha$  in cells expressing S101A-mutant RI $\alpha$  indicates the presence of other phosphorylation sites that are targeted in cells. To address this, two other putative phosphorylation sites within RI $\alpha$  protein, namely Ser<sup>77</sup> and Ser<sup>83</sup>, were investigated (Fig. 2E). These two sites have been shown to be phosphorylated in human heart tissues in the context of ischemic heart disease (20) and thus may contribute to the background signal in our phosphorylation assays. Phosphorylation reactions were performed in HEK293T cells overexpressing FLAG-tagged constructs of either WT or a mutant version of RI $\alpha$  protein with both Ser<sup>77</sup> and Ser<sup>83</sup> mutated to alanine. All reactions were conducted with overexpressed PKGI $\alpha$  either with or without 2 h of cGMP stimulus. In cells overexpressing WT protein, low levels of RI $\alpha$  phosphorylation in cells without cGMP stimulus were observed as seen in previous experiments. However, cells expressing the S77A/S83A double mutation of RI $\alpha$  did not yield phosphorylation signal without cGMP stimulus, indicating that all background signal observed in non-stimulated samples is due to extraneous in-cell phosphorylation of these two particular sites. As before, immunoblots using anti-FLAG antibodies were performed to show equal expression of RI $\alpha$ . These initial phosphorylation experiments performed under both *in vitro* and in-cell conditions have shown that serine 101 in RI $\alpha$  is indeed a *bona fide* phosphorylation target of PKGI $\alpha$  in mammalian cells.

### Mutation at serine 101 in RI $\alpha$ induces PKAc activity without cAMP stimulus *in vitro* and in cells

As mentioned in the introduction, we hypothesized that phosphorylation of Ser<sup>101</sup> would serve to increase PKAc activity in cells. Therefore, we developed mutations at this site in RI $\alpha$ , via site-directed mutagenesis of the serine to either a glutamate residue (S101E) or an alanine residue (S101A), to test whether this modification will weaken IS binding to the active site of PKAc and thus allow the competition of substrates to bind and be phosphorylated. After generating S101E and S101A mutant constructs for recombinant protein purification (full-length bovine RI $\alpha$  (1–379) in pRSET vector) and S101E mutant for mammalian cell transfection (full-length human RI $\alpha$  (1–379) in pcDNA3.1 vector), components of the PepTag<sup>®</sup> phosphorylation assay were used to assess differences in PKAc activation for wildtype *versus* mutant protein under both *in vitro* and in-cell conditions. Under *in vitro* conditions, WT RI $\alpha$  maintains PKAc in an inhibited state without cAMP stimulus; however, both S101E and S101A mutants display high PKAc activity under basal (cAMP-free) condi-

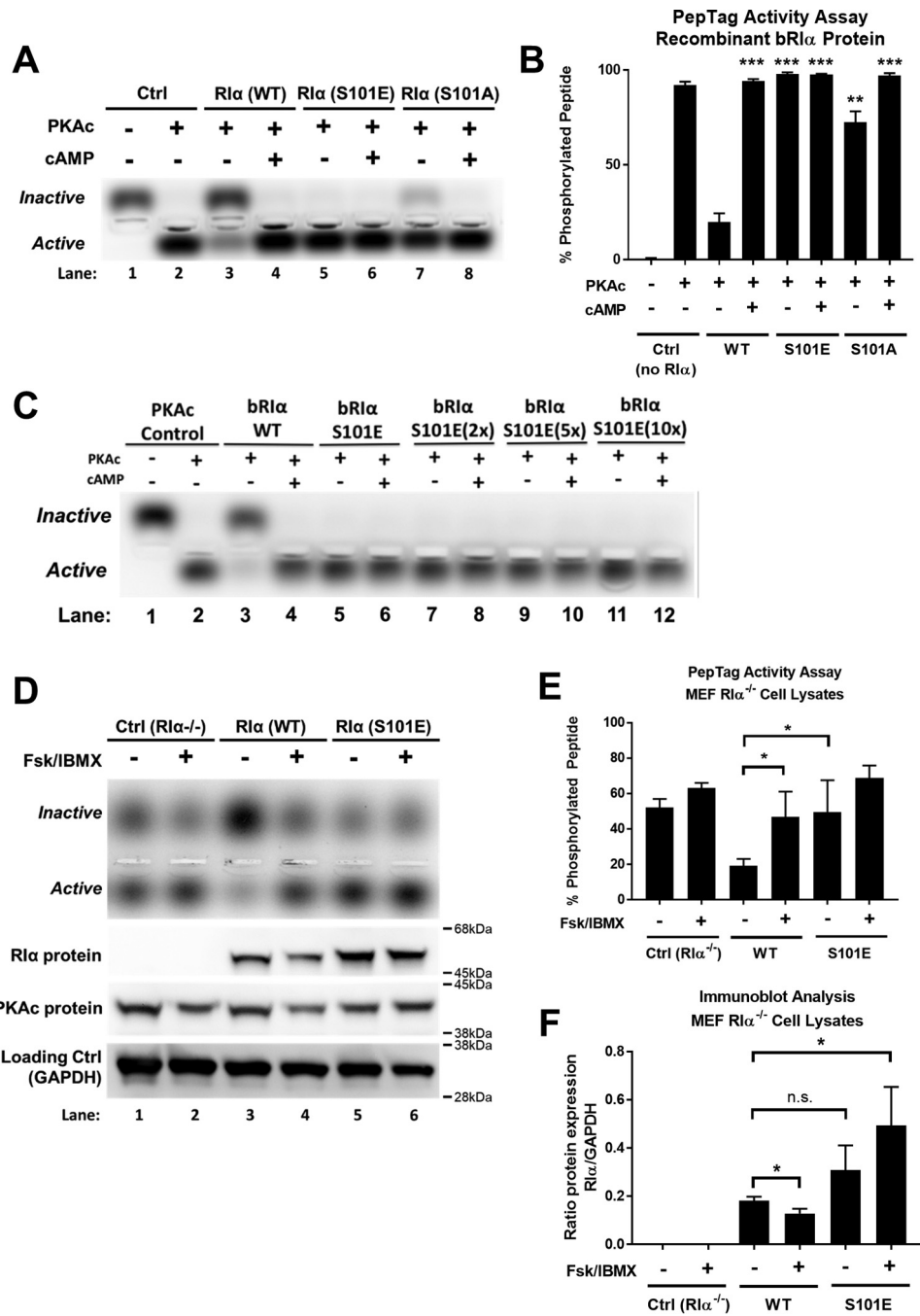
tions (Fig. 3, A and B). Increasing S101E protein concentration from 2 $\times$ , 5 $\times$ , and 10 $\times$  the relative stoichiometry of WT protein had no effect on inhibiting the observed high basal PKAc activity (Fig. 3C).

For in-cell analysis of PKAc activity, WT and S101E-mutant human RI $\alpha$  were overexpressed in MEF RI $\alpha$ <sup>-/-</sup> cells (mouse embryonic fibroblasts with a genetic knockout of *PRKAR1A*). The RI $\alpha$ <sup>-/-</sup> cell line allows for a null background to compare the addition of exogenous RI $\alpha$  overexpression. The relative degree of PKAc phosphorylation activity was compared between non-transfected cells (control RI $\alpha$ <sup>-/-</sup>) and cells overexpressing either WT or S101E-mutant human RI $\alpha$  (Fig. 3, D and E). Control cells displayed high substrate phosphorylation under non-stimulated conditions, thus serving as a positive control for PKAc activity and also confirming earlier observations that in the absence of RI $\alpha$ , PKA activity is not well-regulated even though other R-subunit isoforms are present. In close corroboration with *in vitro* data, overexpression of WT RI $\alpha$  in cells inhibited PKAc activity in the absence of Fsk/IBMX. In contrast to WT, cells expressing the S101E mutant showed high activity without cAMP stimulus, similarly as observed under *in vitro* conditions. We also conducted immunoblot analysis of RI $\alpha$  and PKAc expression in MEF RI $\alpha$ <sup>-/-</sup> lysates for the corresponding activity assay samples examined (Fig. 3, D and F). Two major trends were observed: 1) in WT cells, the level of RI $\alpha$  was slightly decreased upon Fsk/IBMX stimulation; and 2) RI $\alpha$  expression appeared to be slightly higher in S101E cells as compared with cells expressing WT RI $\alpha$  (but only significant when comparing WT vehicle to S101E Fsk/IBMX samples). The competitive nature of the phosphorylation reactions employed suggests that the IS of RI $\alpha$ (S101E) must have a lowered affinity for the PKAc active site cleft; otherwise the PKA substrate would not be able to bind effectively to allow for phosphotransfer activity of PKAc.

Two explanations are possible for the aforementioned results. One possibility is that the RI $\alpha$ (S101E) mutant is simply unable to form a complex with PKAc; alternatively, the mutant RI $\alpha$  protein may still distally bind PKAc (via the R-subunit CNBs domains), even though the modified sequence in the IS region is loosely anchored to the PKAc active site cleft. Therefore, several techniques were explored to evaluate the relative capacity of S101E and S101A mutants of RI $\alpha$  to form holoenzyme.

### Ser<sup>101</sup> mutant variants of RI $\alpha$ pull down PKAc from HEK293T cell lysates via co-immunoprecipitation (co-IP)

To demonstrate that the Ser<sup>101</sup> mutants of RI $\alpha$  can form holoenzyme complexes in cells, FLAG-tagged versions of human RI $\alpha$  were overexpressed in HEK293T cells, whereupon we assessed whether the S101A and S101E mutant variants of RI $\alpha$  were able to pull down overexpressed HA-tagged PKAc protein from cell lysates via co-immunoprecipitation. In this experiment, immunoblotting for the presence of HA-tagged protein in anti-FLAG IP samples can be used to qualitatively determine the state of holoenzyme complex formation in cells. We observed HA-tagged PKAc in the immunoprecipitate of all RI $\alpha$  proteins tested (WT, S101A, and S101E), sig-

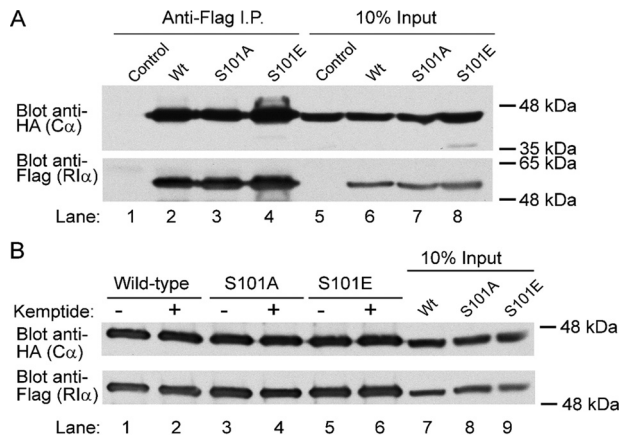


**Figure 3. Mutation of bovine R1α at serine 101 induces PKAc activity without cAMP stimulus in vitro.** *A*, analysis of *in vitro* PKAc kinase activity using the PepTag® activity assay kit, comparing the inhibitory capacity of either WT, phosphomimetic mutant (S101E), or alanine mutant (S101A) variants of purified bovine R1α protein ( $n = 3$ ). *B*, densitometric quantification of three experiments performed as described in *A*, expressed as means  $\pm$  S.D. Statistical significance is indicated as compared with R1α(WT) without cAMP. \*\*,  $p < 0.001$ ; \*\*\*,  $p < 0.0001$ . *C*, qualitative analysis of *in vitro* PKAc kinase activity using the PepTag® activity assay kit, comparing the inhibitory capacity of either WT or phosphomimetic mutant (S101E) variants of purified bovine R1α protein. In this experiment, full-length R1α S101E dimer protein was added in doses of progressively higher molar stoichiometry as compared with PKAc protein. *Lane 1*, no protein control; *lanes 2–12*, 200 nM PKAc; *lanes 3 and 4*, 120 nM WT dimer; *lanes 5 and 6*, 120 nM S101E dimer; *lanes 7 and 8*, 240 nM S101E dimer; *lanes 9 and 10*, 480 nM S101E dimer; *lanes 11 and 12*, 1.2  $\mu$ M S101E dimer ( $n = 1$ ). *D*, analysis of PKAc kinase activity using the PepTag® activity assay kit, comparing WT or phosphomimetic mutant (S101E) variants of human R1α overexpressed in MEF R1α<sup>-/-</sup> cells (mouse embryonic fibroblasts with genetic knockout of *PRKAR1α*). To stimulate cAMP production in these cells, Fsk/IBMX (10 and 100  $\mu$ M) were added for 10 min prior to cell lysis and subsequent activity assay ( $n = 3$ ). The *lower panels* are immunoblots of R1α, PKAc, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) loading control for the given treatment conditions. *E*, densitometric quantification of three activity assay experiments performed as described in *D*, expressed as means  $\pm$  S.D. Statistical significance is indicated as compared with WT without Fsk/IBMX. \*,  $p < 0.05$ . *F*, densitometric quantification of three immunoblot experiments performed as described in *D*, expressed as means  $\pm$  S.D. Statistical significance is indicated as compared with WT without Fsk/IBMX. \*,  $p < 0.05$ . *Ctrl*, control.

nifying that these mutations do not influence the formation of heterodimer complexes between PKAc to R1α (Fig. 4A). Furthermore, co-immunoprecipitation of PKAc was also

possible in the presence of excess substrate (Kemptide), indicating that excess substrate does not promote holoenzyme dissociation (Fig. 4B).

## Rla Ser<sup>101</sup> phosphorylation by PKG activates PKAc in cells



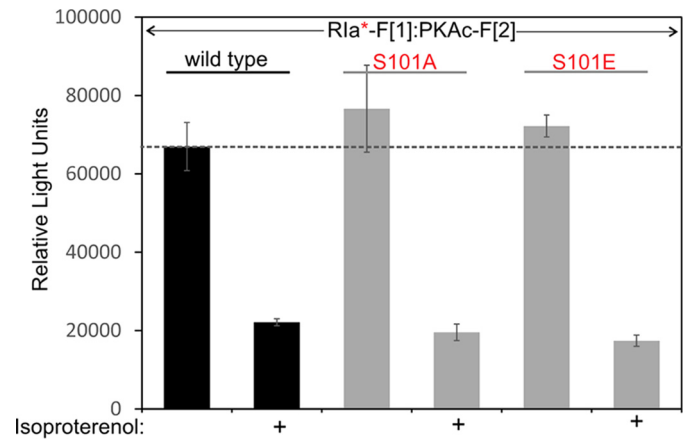
**Figure 4.** Ser<sup>101</sup> mutant variants of Rl $\alpha$  pull down PKAc from HEK293T cell lysates via co-IP. Immunoblot analysis of co-IP reactions from HEK293T cell lysates, comparing cells overexpressing FLAG-tagged versions of either WT or serine 101 mutant variants (S101A and S101E) of Rl $\alpha$ , was performed. The presence of HA-tagged PKAc protein (C $\alpha$ ) was examined in co-IP samples to qualitatively determine the state of Rl $\alpha$ -PKAc complex formation in cells. In *A*, co-IP experiments were performed on untreated lysates, whereas in *B*, co-IP experiments were performed in the presence of 500  $\mu$ M Kempptide substrate peptide. Both S101A and S101E mutants maintained binding to PKAc under all conditions tested, indicating that these mutations do not influence complex formation in cells. *I.P.*, immunoprecipitation.

### Protein-fragment complementation assay (PCA) shows Ser<sup>101</sup> mutants form cAMP-sensitive complexes in cells

To further confirm that mutant Rl $\alpha$  forms stable holoenzyme complex in cells, a bioluminescent PCA (developed by Stefan and co-workers. (27); see “Experimental procedures”) was implemented. This assay uses differential tagging of PKA regulatory and catalytic subunit proteins with fragments of *Renilla* luciferase (RLuc) to create a “split-reporter” system that can be used to monitor R-C complex formation as a function of luciferase-fragment complementation. Upon cellular overexpression of RLuc-tagged versions of PKAc and either wildtype, S101A, or S101E variants of Rl $\alpha$ , the relative degree of Rl $\alpha$ -PKAc protein-protein interaction can be measured in intact cells. Moreover, PKA holoenzyme dissociation can be measured by luminescence signal decrease upon cellular stimulation with isoproterenol (a  $\beta$ -adrenergic receptor agonist known to stimulate cAMP production in cells). We observed that Ser<sup>101</sup> mutants of Rl $\alpha$  still displayed a similar degree of complex formation as compared with wildtype in non-stimulated cells (Fig. 5). Furthermore, bioluminescence signal was diminished upon addition of isoproterenol in all protein constructs tested, thus showing that the Ser<sup>101</sup> mutant variants of Rl $\alpha$ -PKAc complexes are still sensitive cAMP and thus allow for holoenzyme dissociation under stimulatory conditions.

### cAMP response of bRl $\alpha$ proteins shows high inhibitor binding for S101E, but not S101A mutant

For a more quantitative understanding of PKAc binding affinities for wildtype and mutant Rl $\alpha$  subunits *in vitro*, we utilized the ligand-regulated competition (LiReC) fluorescence polarization assay (see “Experimental procedures”) (28). This competitive inhibitor-binding assay is used to detect the relative fluorescence polarization of FAM-IP20, a fluorescein-conjugated PKA inhibitor peptide that is derived from the heat

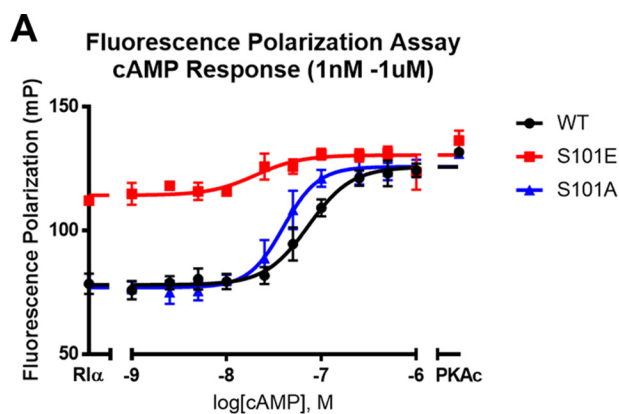


**Figure 5.** PCA shows Ser<sup>101</sup> mutants form cAMP-sensitive complexes in cells. PCA (see “Experimental procedures”) was performed to measure Rl $\alpha$ -PKAc protein association in isoproterenol-stimulated HEK293T cells, comparing WT to mutant Rl $\alpha$  (S101A and S101E). In this assay, increased bioluminescence signal indicates complementation of the luciferase “split reporter” caused by protein-protein interaction mediated by holoenzyme association. Moreover, PKA holoenzyme dissociation is measured by luminescence signal (relative light units) decrease upon cellular stimulation with isoproterenol (a  $\beta$ -adrenergic receptor agonist known to stimulate cAMP production in cells). Shown is a representative of  $n = 3$  independent experiments;  $\pm$  standard deviation from triplicates.

stable protein kinase inhibitor. In the LiReC assay, the relative increase of the fluorescence polarization signal measured as the IP20 probe binds PKAc is indicative of the decrease in anisotropic tumbling of the immobilized inhibitor. First, a cAMP response assay was performed to compare FAM-IP20 binding to PKAc in the presence of either the WT, S101A, and S101E versions of Rl $\alpha$  (Fig. 6). Whereas the S101A mutant behaved similarly as WT protein, the S101E mutant displayed significantly higher levels of FAM-IP20 binding in the absence of cAMP. In this instance, the S101E and S101A mutants showed lower EC<sub>50</sub> values as compared with WT, yet Hill slope values for both mutants were not significantly changed.

### R-subunit inhibition response assay shows S101E binds PKAc with lower relative affinity

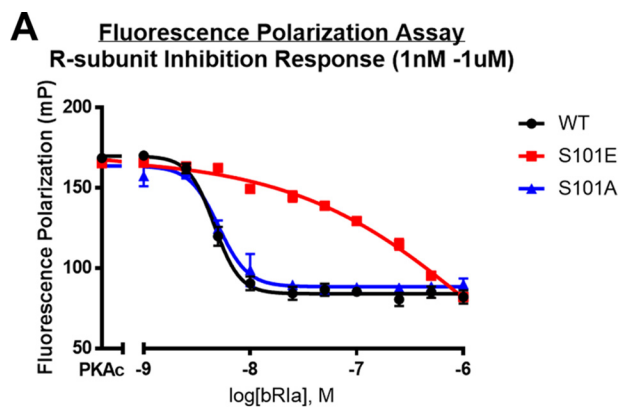
To more conclusively determine whether Rl $\alpha$ (S101E) with its modified linker region can functionally bind PKAc, the assay was modified to assess the inhibitory capacity for the Rl $\alpha$  subunit in the presence of a constant concentration of PKAc (10 nM) and in the absence of cAMP (Fig. 7). WT, S101A, and S101E proteins were compared to discern any differences in formation of holoenzyme complexes for these serine 101 mutants. We observed that significantly higher levels of S101E mutant were required to out-compete FAM-IP20 for binding to PKAc (IC<sub>50</sub><sup>S101E</sup> = 212.5 nM  $\pm$  1.55). In contrast, S101A behaved very similar to WT Rl $\alpha$  (IC<sub>50</sub><sup>WT</sup> = 4.5 nM  $\pm$  1.05; IC<sub>50</sub><sup>S101A</sup> = 5.05 nM  $\pm$  1.08), indicating that both proteins have an affinity for PKAc of less than 10 nM, which is the concentration of PKAc in the assay. These data suggest that the presence of the glutamic acid residue specifically and significantly affects the affinity of the Rl $\alpha$  subunit to the PKAc active site, whereas the more conservative alanine substitution can function similar to unmodified wildtype protein at 10 nM concentrations. Thus, these experiments were able to illustrate that Rl $\alpha$ (S101E) can functionally bind PKAc to out-compete FAM-IP20, but



**B**

bRI $\alpha$ (1-379)	EC <sub>50</sub> (nM)	Hill Slope
WT	72.8 nM +/- 1.1	1.90 +/- 0.21
S101E	20.1nM +/- 1.4	1.80 +/- 0.85
S101A	39.7nM +/- 1.1	2.37 +/- 0.37

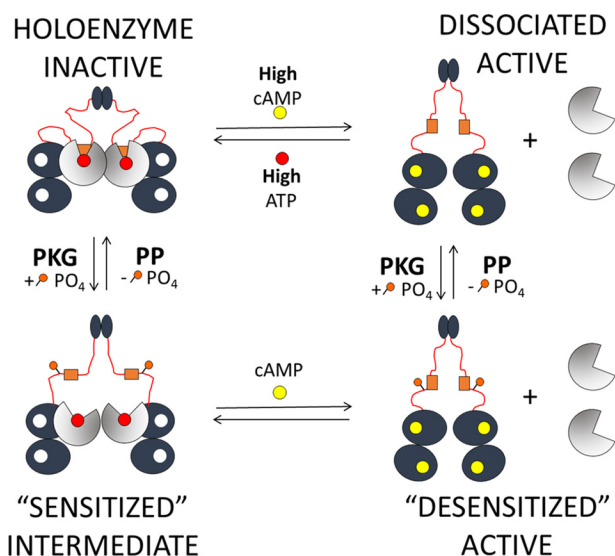
**Figure 6. cAMP response of bRI $\alpha$  proteins shows high inhibitor binding for S101E, but not S101A mutant.** A LiReC fluorescence polarization assay (see “Experimental procedures”) compared cAMP response between WT (black), S101E mutant (red), and S101A mutants (blue) forms of bRI $\alpha$ . A, graph of fluorescence polarization data reported in millipolarization units (mP). B, table of EC<sub>50</sub> values and Hill slope. (Each condition was performed in quadruplicate, *n* = 4; error bars and table data are representative of  $\pm$  S.E.).



**B**

bRI $\alpha$ (1-379)	IC <sub>50</sub> (nM)
WT	4.5 nM +/- 1.05
S101E	212.5nM +/- 1.55
S101A	5.05nM +/- 1.08

**Figure 7. R-subunit inhibition response assay shows that S101E binds PKAc with lower relative affinity.** LiReC fluorescence polarization assay (see “Experimental procedures”) compared inhibitory capacity of regulatory subunit protein between WT (black), S101E mutant (red), and S101A mutant (blue) forms of bRI $\alpha$ . A, graph of fluorescence polarization data reported in millipolarization units (mP). B, table of IC<sub>50</sub> values and Hill slope. (Each condition was performed in quadruplicate, *n* = 4; error bars and table data are representative of  $\pm$  S.E.).



**Figure 8. PKG phosphorylation model of type I PKA activation.** A cartoon diagram of a modified, three-state model of type I PKA holoenzyme activation based on RI $\alpha$  phosphorylation by PKG is shown. Our model illustrates that type I holoenzyme may not transition directly from an inactive holoenzyme-complex state to a completely dissociated and active state. Instead, our data suggest that phosphorylation of serine 101 in RI $\alpha$  lowers the affinity between the IS of RI $\alpha$  and the active site of PKAc, thus leading to a “sensitized intermediate” state that displays PKAc catalytic activity while maintaining a holoenzyme complex configuration. This intermediate would then respond to physiological levels of cAMP toward a “desensitized” active state of the dissociated holoenzyme complex. Our model also denotes the potential for reversibility of linker region phosphorylation by protein phosphatases (PP).

the relative inhibitory equilibrium constant was approximately 2 orders of magnitude higher compared with the wildtype RI $\alpha$  control.

**Discussion**

To summarize the work presented here, we have revisited and additionally built upon a former concept within the field of protein kinase signaling, which highlights a potential cross-talk mechanism between the key contributors of cyclic nucleotide signaling in eukaryotic cells (PKA and PKG). Our evidence has validated that Ser<sup>101</sup> in RI $\alpha$  is a target of PKG both *in vitro* and in cells, and furthermore we showed that introduction of a phosphorylation modification (or phosphomimetic mutation) at the serine 101 site in RI $\alpha$  leads to a state of heightened PKAc activity while still maintaining a RI $\alpha$ –PKAc complex. These data significantly shift the paradigm regarding our understanding of Type I PKA signaling, in that modification of the RI $\alpha$  linker region can serve to circumvent previously described kinetic restrictions of holoenzyme dissociation and thus trigger what we denote as “desensitized” PKAc activity. We have thus created a revised model of RI $\alpha$  holoenzyme activation to better illustrate how the effect of PKG phosphorylation could lead to significant changes in the equilibrium of RI $\alpha$ –PKAc association and dissociation (Fig. 8). In this model, modification of RI $\alpha$  leads a “sensitized intermediate” state of the RI $\alpha$  holoenzyme that is capable of phosphotransfer activity because of the lack of linker region accessibility at the PKAc active site. The addition of physiological levels of cAMP thus can trigger rapid dissociation of the holoenzyme complex, therefore creating a “desensitized” state of activity because of the reduced affinity of the modified



## R1a Ser<sup>101</sup> phosphorylation by PKG activates PKAc in cells

RI $\alpha$  to reassociate with PKAc. Although this study focused upon PKG phosphorylation of Ser<sup>101</sup> in RI $\alpha$ , our supplementary investigation of alternative phosphorylation sites in the linker region portion of the protein (*i.e.* Ser<sup>77</sup> and Ser<sup>83</sup>) has further highlighted the potential significance of linker region modification as a regulatory mechanism for type I PKA in cells. Because these sites are differentially phosphorylated in heart tissues with and without onset of heart failure (20), it is possible that these linker region phosphorylation sites in RI $\alpha$  are specifically involved in regulating response to stress in the context of cardiac disease. Further investigation is also required to address the presumed role of protein phosphatases in allowing reversibility of phosphorylation at these linker region sites.

In terms of chemical equilibrium, we propose that modified RI $\alpha$  (*i.e.* the sensitized intermediate) has a significant (nearly 2 orders of magnitude) decrease in binding affinity to PKAc as compared with the 0.1 nM  $K_d$  of the inactive holoenzyme. This assertion could very well explain discrepancies observed in related scientific literature, where it has been shown that elevation of cAMP under physiological conditions may not be sufficient to explain how PKA is activated upon stimulatory signaling in the cell (29). However, further examination is required to determine the exact nature of binding in the “sensitized intermediate” protein complex, with particular regard to 1) pseudo-substrate binding/accessibility to the active site cleft of PKAc, and 2) effects upon allosteric interactions in the modified holoenzyme. In future studies, surface plasmon resonance methods will be used to quantitatively determine the binding affinity of Ser<sup>101</sup> mutants for PKAc. Utilization of alternative biophysical methodologies such as hydrogen/deuterium exchange mass spectrometry could also help to better explore the relative degree of conformational dynamics for WT and S101E proteins binding to PKAc by quantifying the degree of solvent exposure upon differing timescales of protein–protein interactions.

Although this study provides additional biochemical characterization of PKG phosphorylation of Ser<sup>101</sup> in RI $\alpha$  in a cellular overexpression system, a final goal for this work is to assess the significance of this mechanism within *in vivo* systems. Because both PKG and PKA signaling play critical roles within cardiac and smooth muscle cell physiology (26, 30, 31), our ongoing research is aimed at detecting endogenous phosphorylation of RI $\alpha$  in differentiated muscle cell types, either treated with or without stimulus to drive PKG activity. Along this line of investigation, determining the role of oxidative stress signaling in the activation of this cross-talk mechanism will be of particular importance, because reactive nitrogen/oxygen signaling has been shown to result in alterations of heart physiology as manifested through both PKA and PKG (32–34). Given that both PKG and PKA have been implicated as targets in disease conditions such as dilated cardiomyopathy (35–37), heart failure with preserved ejection fractions (38, 39), and ischemia reperfusion injury (40, 41), characterization of RI $\alpha$  phosphorylation within endogenous tissues may further bolster the significance of this mechanism within the context of clinically relevant cardiovascular diseases.

## Experimental procedures

### Materials

Antibodies for PKAc and RI $\alpha$  were from BD Biosciences (San Jose, CA); antibodies for glyceraldehyde-3-phosphate dehydrogenase were from Santa Cruz Biotechnology (Dallas, TX). Anti-HA and anti-species secondary antibodies were obtained from Santa Cruz Biotechnology. Other antibodies, chemicals, and reagents were obtained from Sigma unless otherwise stated.

### Recombinant DNA and site-directed mutagenesis

Constructs of full-length human RI $\alpha$  gene (hRI $\alpha$  1–379) incorporated into the pCDNA3.3 mammalian expression vector were used for cellular transfection experiments in MEFs. Point mutations (hRI $\alpha$ [S101A] and hRI $\alpha$ [S101E]) were introduced by site-directed mutagenesis via QuikChange<sup>®</sup> PCR as per the manufacturer’s instructions (Stratagene, La Jolla, CA). The same genetic material was also incorporated into pRSET-Xa3 vector for bacterial recombinant protein expression. Point mutations (bRI $\alpha$ [S101A] and bRI $\alpha$ [S101E]) were introduced into both constructs by site-directed mutagenesis via QuikChange<sup>®</sup> PCR as per the manufacturer’s instructions (Stratagene). FLAG-tagged RI $\alpha$  was constructed by PCR using an untagged RI $\alpha$  vector as a template. The PCR product was digested and inserted into the pFLAG-D expression vector. The S101A and S101E mutant RI $\alpha$  constructs were generated using overlapping extension PCR. All constructs that went through a PCR step were sequenced to ensure that the expected coding sequence was present.

### Expression and purification of recombinant proteins from *Escherichia coli*

The C-subunit was expressed in *E. coli* and purified as described (42). Full-length (1–379) constructs of either wild-type, S101E, and S101A mutants of bRI $\alpha$  (in pRSET-Xa3 vector) were expressed in BL21(DE3) cells and purified by cAMP affinity chromatography in 20 mM MES (pH 6.5), 100 mM NaCl, 2 mM EGTA, 2 mM EDTA, and 5 mM DTT. RI $\alpha$  proteins were further purified on a gel filtration column using Superdex 200 and concentrated for purposes of biochemical assays using Amicon<sup>®</sup> centrifugal filters (EMD Millipore, Billerica, MA).

### *In vitro* PKA RI $\alpha$ phosphorylation by PKGI $\alpha$

To examine RI $\alpha$ –PKAc holoenzyme phosphorylation by PKGI $\alpha$ , purified PKGI $\alpha$  (43) was added to a 20- $\mu$ l reaction mix containing 0.6  $\mu$ g of wildtype RI $\alpha$ –PKAc holoenzyme complex, 30 mM HEPES (pH 7.4), 10 mM MgCl<sub>2</sub>, 10 mM  $\beta$ -glycerol phosphate, 100 mM ATP, 0.05 mCi of [ $\gamma$ -<sup>32</sup>P]ATP, and 1  $\mu$ M of the indicated cyclic nucleotides. The reactions were incubated at 30 °C for 2 h. The reactions were terminated by adding 20  $\mu$ l of 2 $\times$  SDS-PAGE loading buffer containing 100 mM EDTA followed by boiling. Phosphate incorporation was determined by SDS-PAGE/autoradiography. Densitometric analysis was performed using ImageJ.

### Cell culture: Growth, transfection, and treatments

HEK293T and mouse embryonic fibroblast (MEF) cells were used throughout this study. Propagating cultures were

grown in 10-cm dishes using Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. MEF cultures contained 1% penicillin/streptomycin, unless otherwise stated.

#### Phosphorylation of R1 $\alpha$ in HEK293T cells

HEK293T cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum in a 37 °C incubator with a 5% CO<sub>2</sub> atmosphere. The day before transfection, the cells were split into a 6-well cluster dish such that the cells were 90–95% confluent at the time of transfection. The cells were transfected with the indicated expression constructs with Lipofectamine 2000 using conditions recommended by the manufacturer (Thermo Fisher). The next day, the medium was removed, and 1 ml of phosphate-free media and 100  $\mu$ Ci of <sup>32</sup>PO<sub>4</sub> was added to each well. The cells were incubated at 37 °C for 2 h, and then 250  $\mu$ M 8-CPT-cGMP was added to the appropriate wells. The cells were incubated for an additional 2 h at 37 °C, at which point the medium was removed, the cells were washed once with ice-cold PBS, and the cells were lysed on the plate in PBS, 0.1% Nonidet P-40, 1 $\times$  protease inhibitor mixture (Calbiochem, San Diego, CA), and 1 $\times$  HALT phosphatase inhibitor (Cell Signaling Technology, Danvers, MA). Cleared lysates were added to 20  $\mu$ l of anti-FLAG affinity gel and incubated for 1 h at 4 °C with constant mixing. The beads were washed and boiled in 30  $\mu$ l of 1 $\times$  SDS sample buffer, and phosphorylation was analyzed by SDS-PAGE/autoradiography. The relative amount of immunoprecipitated FLAG-tagged R1 $\alpha$  was determined by immunoblotting.

#### PepTag<sup>®</sup> non-radioactive PKA phosphorylation assay

Assays using both cell lysates and recombinant proteins were performed in a modified procedure from the manufacturer's directions. For cell-based experiments, MEF cell samples were plated on 6-well dishes at passage 3. Transfections were conducted using Lipofectamine 2000<sup>®</sup> (Thermo Fisher) as per the manufacturer's directions. For activity experiments, MEF cells were serum-starved for 2 h before conducting 10 min of Fsk/IBMX stress treatment (*i.e.* 20  $\mu$ M forskolin and 100  $\mu$ M IBMX). 10  $\mu$ l of cell lysate (of normalized protein concentration) was added to 5  $\mu$ l each of "5 $\times$  peptide," "5 $\times$  buffer," and distilled water for all reactions. The samples were mixed and incubated at room temperature for 30 min and then heat-inactivated at 95 °C for 10 min. For recombinant protein experiments: R1 $\alpha$  and PKAc proteins were incubated *in situ* at a 1.2:1 molar stoichiometry within a 10- $\mu$ l volume (for R1 $\alpha$ (1–379) dimer protein, 5  $\mu$ l of 1.2  $\mu$ M R-subunit was added to 5  $\mu$ l of 2  $\mu$ M PKAc). Then 5  $\mu$ l each of 5 $\times$  peptide, 5 $\times$  buffer, and either 5 mM cAMP or distilled water was added to start the reactions. Samples were mixed and incubated at room temperature for 30 min and then heat-inactivated at 95 °C for 10 min. For all experiments, phosphopeptides were separated by 0.8% agarose gel as per the manufacturer's directions. Densitometric analysis of gel images was performed using ImageJ. Activity is expressed as the percentage of phosphorylated peptide, calculated by the ratio of phosphorylated peptide signal divided by total signal.

#### Immunoblotting

SDS-PAGE samples were prepared using 5 $\times$  Laemmli buffer (100 mM Tris-HCl, pH 7.0, 10% SDS, 50% glycerol, 0.01% bromphenol blue). Protein concentrations were normalized between samples, and then SDS-PAGE was performed using NuPAGE 4–12% gradient gels (either 10-, 12-, or 15-well) in MES running buffer (55 mM MES, 10 mM Tris-base, 1 mM EDTA, 0.1% SDS). The gels were resolved at 150 V for 70 min and then subsequently transferred to 0.45- $\mu$ m nitrocellulose membranes (200 mA/blot for 60 min). The membranes were blocked using 5% milk protein (or with 5% BSA for phosphoblots) in 1 $\times$  PBS, 0.05% Tween for 60 min at room temperature, and then primary antibodies were incubated overnight at 4 °C in 1 $\times$  PBS, 0.05% Tween 20 or 1 $\times$  TBS, 0.1% Tween 20 at the concentrations specified by the manufacturer. Densitometric analysis was performed using ImageJ.

#### Co-immunoprecipitation

Expression vectors encoding HA-tagged PKAc and WT or Ser<sup>101</sup> mutant FLAG-tagged R1 $\alpha$ , or empty vector were transfected into 293T cells using Lipofectamine 2000. After 20 h, the cells were lysed in PBS, 0.1% Nonidet P-40 with 1 $\times$  protease inhibitor mixture. The lysates were cleared by centrifugation at 16,000  $\times$  g for 10 min, and supernatants were incubated with anti-FLAG M2 affinity gel for 1 h at 4 °C with constant mixing. The beads were washed three times with PBS, 0.1% Nonidet P-40, proteins were eluted by boiling in 2 $\times$  Laemmli buffer, and bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting. For some experiments, 500  $\mu$ M Kemptide was added during the immunoprecipitations.

#### PCA using fragmented RLuc

Plasmid constructs of PKAc tagged with F1 fragment (PKAc-F1) and R1 $\alpha$  tagged with F2 fragment (R1 $\alpha$ -F2) were generated previously (27). S101E and S101A mutations of R1 $\alpha$  were introduced into R1 $\alpha$ -F2 by site-directed mutagenesis via QuikChange<sup>®</sup> PCR as per the manufacturer's instructions (Stratagene). PKAc-F1 and either WT, S101E, or S101A versions of R1 $\alpha$ -F2 plasmids were co-transfected into HEK293T cells, and bioluminescence signal was compared under basal conditions, as well as with 15 min of 10 nM isoproterenol stimulus.

#### LiReC fluorescence polarization assay

Use of the LiReC assay has been described previously (28). All reactions were conducted in assay buffer (50 mM MOPS, pH 7.0, 35 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM ATP, 2 mM DTT, and 0.005% Triton X-100). End-point fluorescence polarization measurements were performed using a GENios Pro<sup>®</sup> plate-reader spectrometer (Tecan, Männedorf, Switzerland), configured with 485-nm excitation and 535-nm emission filters and using optimal gain settings. Concentrations of proteins, [5/6-FAM]-IP20, and cAMP for each experimental method are listed below: for cAMP response experiments, 12.0 nM PKA catalytic subunit, 14.4 nM PKA regulatory subunit bR1 $\alpha$  (91–379) or bR1 $\alpha$  (1–379) (based on monomer concentration), 2.0 nM [5/6-

## Rla Ser<sup>101</sup> phosphorylation by PKG activates PKAc in cells

FAM]-IP20, 1 nM-1  $\mu$ M cAMP (dissolved in 1 $\times$  buffer without ATP or DTT); and for R-subunit inhibition response experiments, 10.0 nM PKA catalytic subunit, 1 nM-1  $\mu$ M PKA regulatory subunit bRI $\alpha$ (91–379) or bRI $\alpha$ (1–379) (based on monomer concentration), 1.67 nM [5/6-FAM]-IP20.

### Statistics

All activity, immunoblot, and PCA data are presented as means  $\pm$  S.D., and all fluorescence polarization assay data are presented as means  $\pm$  S.E. GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, CA) was used for all statistical analysis. Statistical analyses were performed by unpaired Student's *t* test of planned comparisons.

**Author contributions**—K. J. H. designed and conducted most of the experiments, built expression constructs, generated recombinant proteins, analyzed the results, and wrote most of the paper. D. E. C. built expression constructs, conducted radioactive phosphorylation and co-immunoprecipitation experiments, and analyzed results. A. R. conducted protein complementation assay experiments and analyzed results. E. S. conducted protein complementation assay experiments, analyzed results, and provided materials for the study. H. H. P. provided material and facilities for the study and wrote the paper with K. J. H. S. S. T. conceived the idea for the project, provided material and facilities for the study, and wrote the paper with K. J. H.

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