

# $\alpha$ -Actinin is a potent regulator of G protein-coupled receptor kinase activity and substrate specificity in vitro

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**Abstract** G protein-coupled receptor kinases (GRKs) phosphorylate G protein-coupled receptors, thereby terminating receptor signaling. Herein we report that  $\alpha$ -actinin potently inhibits all GRK family members. In addition, calcium-bound calmodulin and phosphatidylinositol 4,5-bisphosphate (PIP2), two regulators of GRK activity, coordinate with  $\alpha$ -actinin to modulate substrate specificity of the GRKs. In the presence of calmodulin and  $\alpha$ -actinin, GRK5 phosphorylates soluble, but not membrane-incorporated substrates. In contrast, in the presence of PIP2 and  $\alpha$ -actinin, GRK5 phosphorylates membrane-incorporated, but not soluble substrates. Thus, modulation of  $\alpha$ -actinin-mediated inhibition of GRKs by PIP2 and calmodulin has profound effects on both GRK activity and substrate specificity.

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**Key words:** G protein-coupled receptor; G protein-coupled receptor kinase; Cytoskeleton; Phosphatidylinositol phosphate; Calmodulin; Enzyme regulation

## 1. Introduction

The rapid response of most G protein-coupled receptors (GPCRs) to an agonist is closely followed by the recruitment of G protein-coupled receptor kinases (GRKs). GRKs are divided into three subfamilies based on sequence and functional similarities: GRK1 (rhodopsin kinase), GRK2 and 3 ( $\beta$ -adrenergic receptor kinases 1 and 2) and GRK4, 5 and 6. The GRK family is well characterized for its ability to phosphorylate GPCR and terminate traditional signaling cascades by homologous desensitization. GRK phosphorylation of most GPCRs recruits an arrestin family member which binds the GPCR and initiates its internalization [1].

Additional recently elucidated roles for GRKs demonstrate that these kinases are not simply terminators of signaling, but

also can initiate signal transduction. GPCRs can activate the mitogen-activated protein kinase pathway through a G protein  $\beta\gamma$ -dependent activation of Ras [2]. Additionally, Premont et al. [3], recently identified a GRK interacting protein (GIT1) with ADP-ribosylation factor-GTPase activation protein (ARF-GAP) activity. Overexpression of GIT1 has profound effects on the signaling mediated through a prototypical GPCR, the  $\beta_2$ -adrenergic receptor ( $\beta_2$ AR). The cellular effects of GIT1 are dependent on the ARF-GAP domain of this protein, therefore suggesting that GRKs may serve to integrate signals from GPCRs to the small GTPase ARF. The finding that GRK2 is a tubulin kinase [4–6] further suggests roles for GRKs beyond simply terminating GPCR signaling. The identification of tubulin as a non-receptor, soluble substrate of GRK2 also suggests that there may be additional unidentified substrates for each of the GRKs.

The potential pleiotropic roles of the GRKs suggest that it is necessary that kinase activity be tightly regulated. GRK-mediated phosphorylation is known to be regulated by phosphatidylinositol 4,5-bisphosphate (PIP2), calmodulin and actin. PIP2 enhances GRK-mediated phosphorylation of GPCRs by co-localizing kinase and substrate [1]. Conversely, calmodulin interferes with GRK translocation to membranes [7], thereby inhibiting GRK-mediated phosphorylation of GPCRs without affecting the phosphorylation of soluble substrates such as casein. Recently, we characterized actin as a novel inhibitor of GRK5 function. In contrast to PIP2 and calmodulin, which modulate only membrane localization and thus GPCR phosphorylation, actin directly inhibits GRK-mediated phosphorylation of both soluble and membrane-incorporated substrates [8].

During our characterization of the actin-mediated inhibition of GRK5, we discovered another inhibitor of GRK5, the actin binding protein  $\alpha$ -actinin.  $\alpha$ -Actinin is a member of the spectrin superfamily of actin crosslinking proteins. Both spectrin and  $\alpha$ -actinin are found in most cell types.  $\alpha$ -Actinin is most abundant in muscle cells, while spectrin (also known as fodrin) is a major component of erythrocyte membranes and comprises 3% of the total particulate protein in brain tissue [9]. In addition to binding and crosslinking actin,  $\alpha$ -actinin and spectrin also bind lipid, thus creating a submembrane meshwork of crosslinked actin. Spectrin and  $\alpha$ -actinin also bind various ion channels, cell adhesion molecules and transmembrane receptors and incorporate them into this actin network to create specialized signaling domains [9]. Here, we demonstrate that  $\alpha$ -actinin is a potent inhibitor of

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**Abbreviations:** GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; ARF-GAP, ADP-ribosylation factor-GTPase activation protein; PIP2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidyl serine; PKA, protein kinase A;  $\beta_2$ AR,  $\beta_2$ -adrenergic receptor

all members of the GRK family and identify a regulatory mechanism whereby  $\alpha$ -actinin modulates both the activity and substrate specificity of these enzymes.

## 2. Materials and methods

### 2.1. Materials

Bovine milk  $\beta$ -casein and phosphatidyl serine (PS) (Sigma); bovine brain calmodulin (Calbiochem); PIP2 (Boehringer Mannheim) and protein kinase A (PKA) (Promega) were purchased for these studies. GRK1, GRK2 and GRK5 were purified from Sf9 insect cells as previously described [10–12]. Chicken gizzard (smooth muscle)  $\alpha$ -actinin was either purchased from Sigma or purified as previously described [13]. Similar results were obtained when using both preparations. Spectrin was purified as previously described and dialyzed into 10 mM phosphate buffer, pH 7.4, 50 mM NaBr, 1 mM NaN<sub>3</sub>, 0.05% Tween and 1 mM DTT [14]. Profilin was a generous gift from Don Kaiser and Tom Pollard. Purified peptide (RRREEEEE-SAAA) was prepared in Tris, adjusting the pH to 7.2. Rod outer segment membranes containing rhodopsin were purified as previously described [15].  $\beta_2$ AR was purified and reconstituted in either 5% PIP2/95% PS or 100% PS as previously described [16,17].

### 2.2. Phosphorylation of protein substrates

GRK or PKA, normalized for equivalent casein kinase activity, and the indicated concentration of actin binding proteins were incubated with either rod outer segments containing rhodopsin,  $\beta_2$ AR (3 pmol) or casein (20  $\mu$ g) at 30°C for 5, 10 or 15 min respectively, in 20 mM Tris, pH 7.5, 10 mM MgCl<sub>2</sub>, 2 mM EDTA, 1 mM DTT and 60  $\mu$ M ATP (1  $\mu$ Ci). Reactions examining the effect of calmodulin contained 2 mM CaCl<sub>2</sub>, which in itself does not alter kinase activity. For GPCR or casein phosphorylation, 25  $\mu$ l of sample loading buffer (100 mM

Tris, pH 7.2, 4% SDS, 200 mM DTT, 0.25% pyronin Y, 20% glycerol) was added to terminate the 25  $\mu$ l reaction and the samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The gels were dried and exposed to a PhosphorImager screen (Molecular Dynamics). The amount of phosphorylation was quantitated using Image Quant and the mean of at least three independent experiments is expressed as a percent of substrate phosphorylation in the absence of any added inhibitors.

### 2.3. GRK5-mediated phosphorylation of a soluble synthetic peptide substrate

GRK5-mediated phosphorylation of peptide substrate was determined by incubating peptide (1 mM) and purified GRK5 (10 nM) as previously described [18]. Reactions were applied to P-81 phosphocellulose paper, washed free of ATP and quantitated using scintillation counting.

### 2.4. Curve fitting and data analysis

Inhibition constants and curve fits for data shown in Figs. 1 and 2 were obtained using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

## 3. Results and discussion

We recently reported that actin binds and inhibits GRK5 [8]. We sought to determine if other known actin binding proteins could relieve this inhibition. Surprisingly, addition of  $\alpha$ -actinin, rather than relieving actin-mediated inhibition of GRK5, resulted in further GRK5 inhibition (data not shown). Subsequent experiments demonstrated that  $\alpha$ -actinin alone inhibits GRK5-mediated casein phosphorylation (Fig.

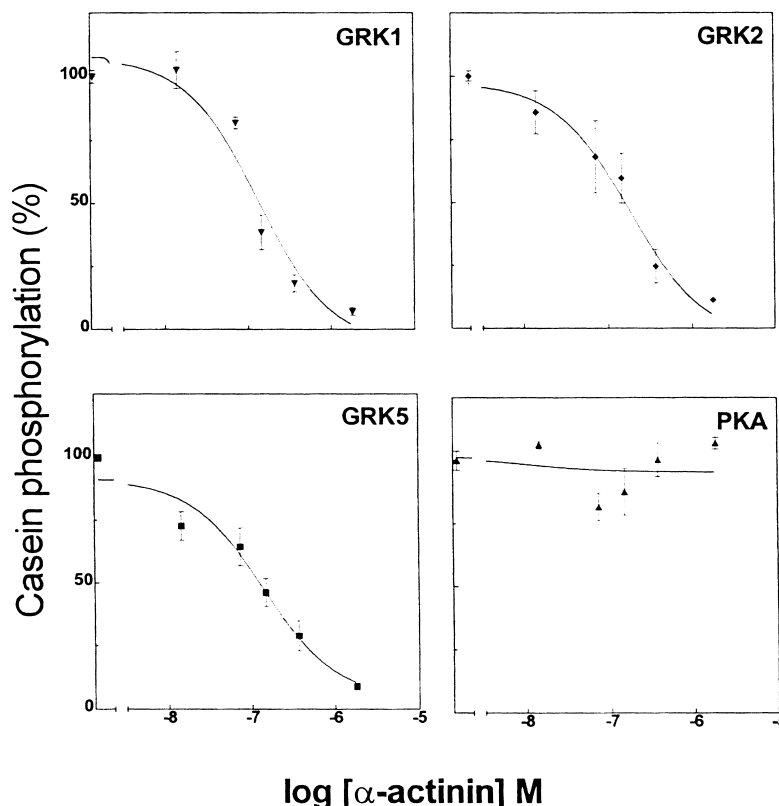


Fig. 1.  $\alpha$ -Actinin is a GRK-specific inhibitor. Equivalent casein kinase activities of the four kinases (0.4–2.4  $\mu$ M), GRK1, 2, 5 and PKA, were incubated with casein and increasing concentrations of  $\alpha$ -actinin in 60  $\mu$ M [<sup>32</sup>P] $\gamma$ -ATP (1  $\mu$ Ci), 20 mM Tris, pH 7.5, 10 mM MgCl<sub>2</sub>, 2 mM EDTA and 1 mM DTT for 15 min at 30°C as described in Section 2. The mean and S.E.M. of at least three experiments are plotted as percent inhibition of casein phosphorylation, with 100% representing the phosphorylation observed in the absence of inhibitor. The IC<sub>50</sub>s for  $\alpha$ -actinin inhibition are 142 nM  $\pm$  35 for GRK1, 177 nM  $\pm$  134 for GRK2 and 139 nM  $\pm$  72 for GRK5.

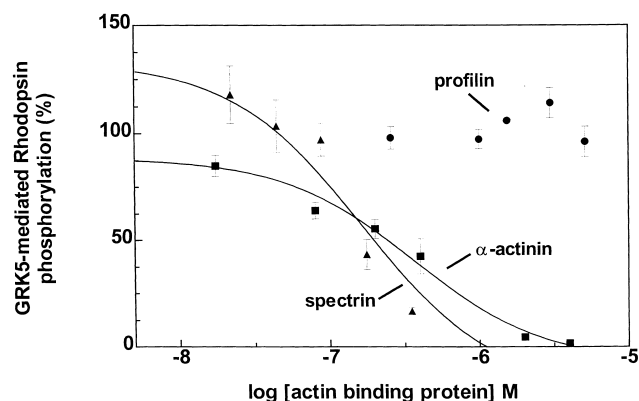


Fig. 2. GRK5 inhibition is specific to the spectrin family of actin binding proteins. The indicated concentration of spectrin family members,  $\alpha$ -actinin (squares) and spectrin (triangles) or the unrelated actin binding protein, profilin (circles), was added to GRK5 and bovine rod outer segments incubated for 5 min under light at 30°C as described in Section 2. The mean and S.E.M. of at least three independent experiments are shown. The  $IC_{50}$ s for spectrin family proteins are  $258 \text{ nM} \pm 148$  for  $\alpha$ -actinin inhibition and  $320 \text{ nM} \pm 123$  for spectrin.

1, GRK5 panel). To determine if  $\alpha$ -actinin inhibits other GRKs as well, we incubated increasing concentrations of chicken gizzard  $\alpha$ -actinin with a GRK representative of each of the GRK subfamilies. Data represented in Fig. 1 demonstrate that GRK1, GRK2 and GRK5 are all potently inhibited by  $\alpha$ -actinin. In contrast, casein phosphorylation by PKA, another serine–threonine kinase, is not affected. The  $IC_{50}$  values for  $\alpha$ -actinin inhibition of casein phosphorylation by each GRK are identical ( $IC_{50}$ s around 150 nM) and are similar to the binding affinities observed for other  $\alpha$ -actinin binding proteins, such as the NMDA receptor [19]. This suggests that  $\alpha$ -actinin, unlike calmodulin and actin, inhibits all GRK subfamilies equally. Therefore,  $\alpha$ -actinin probably binds to a region that is conserved among all the GRKs but not found in other kinases such as PKA.

$\alpha$ -Actinin and spectrin are closely related both by primary sequence and actin binding activities (reviewed in [20]) leading us to speculate that spectrin may also regulate GRK5 activity. This prediction is borne out in the experiments illustrated in Fig. 2 where GRK5-mediated rhodopsin phosphorylation is measured in the presence of increasing concentrations of  $\alpha$ -actinin, spectrin and profilin. Both  $\alpha$ -actinin and spectrin inhibit GRK5-mediated phosphorylation of the GPCR, rhodopsin, while the unrelated actin binding protein, profilin, has no effect (Fig. 2).  $\alpha$ -Actinin and spectrin have similar  $IC_{50}$  values, indicating that these proteins are equally potent inhibitors of GRK5. The  $IC_{50}$  values for inhibition of soluble casein phosphorylation (Fig. 1) and membrane-bound rhodopsin phosphorylation (Fig. 2) by  $\alpha$ -actinin are also identical within experimental error, demonstrating that  $\alpha$ -actinin directly inhibits GRK catalytic activity, rather than specifically interfering with the membrane association of these enzymes.

We chose GRK5 as the representative GRK to characterize the effects of calmodulin and PIP2 on  $\alpha$ -actinin-mediated regulation. GRK5 has been extensively studied with regards to calmodulin and PIP2 regulation and the binding sites on GRK5 have been mapped [1,7]. Several groups have established that calmodulin inhibits GRK5-mediated phosphorylation of membrane-bound receptor substrates (reviewed by

Pitcher et al. [1]). Pronin et al. demonstrated that calmodulin interferes with the ability of GRK5 to recognize the receptor substrate in a lipid environment [7]. To determine if calmodulin has an effect on  $\alpha$ -actinin inhibition of GRK5, we assayed GRK5-mediated phosphorylation of membrane-bound and soluble substrates in the presence of  $\alpha$ -actinin and 0.1 or 1.0  $\mu\text{M}$  calcium-bound calmodulin.  $\alpha$ -Actinin inhibition of GRK5-mediated soluble substrate (casein) phosphorylation is reversed by calmodulin in a dose-related way (Fig. 3, upper panel). In contrast, addition of calmodulin along with  $\alpha$ -actinin further reduces  $\beta_2\text{AR}$  phosphorylation in a dose-dependent manner (Fig. 3, lower panel, compare columns 3 and 4 with column 2). This result agrees with previously reported findings that calmodulin inhibits GRK-mediated phosphorylation of other membrane-bound GPCRs [7]. Thus, calmodulin binding to GRK5 provides a means of relieving  $\alpha$ -actinin inhibition to allow for soluble substrate phosphorylation while simultaneously inhibiting membrane substrate phosphorylation.

We next examined the effects of PIP2 on  $\alpha$ -actinin-mediated inhibition of GRK5 (Fig. 4). PIP2 binds to both  $\alpha$ -actinin and GRKs [1,21]. Unlike calmodulin which relieves  $\alpha$ -actinin inhibition of soluble substrate phosphorylation, addition of PIP2 (shaded columns) does not alter  $\alpha$ -actinin inhibition of GRK5-mediated soluble peptide phosphorylation (Fig. 4, upper panel). Similar results were obtained using casein as a soluble substrate (data not shown). To determine if PIP2 modulates  $\alpha$ -actinin-mediated inhibition of  $\beta_2\text{AR}$ , a prototypical GPCR,  $\beta_2\text{AR}$  was reconstituted in the presence (Fig. 4, lower panel, shaded columns) or absence (Fig. 4, lower panel, unshaded columns) of 5% PIP2 and GRK5-mediated phosphorylation was quantitated. Addition of  $\alpha$ -actinin to reactions with  $\beta_2\text{AR}$  reconstituted in vesicles without PIP2 (Fig. 4, lower panel, unshaded columns), potentially inhibits GRK5-mediated  $\beta_2\text{AR}$  phosphorylation. In contrast to what is ob-

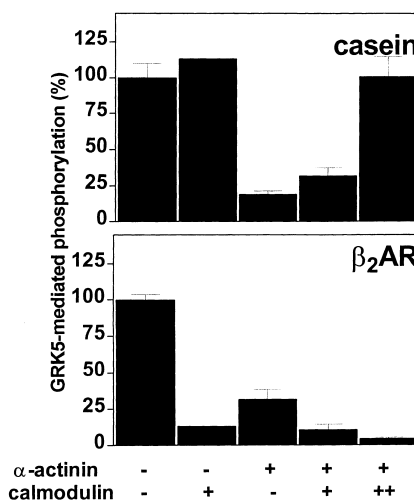


Fig. 3. Calmodulin relieves  $\alpha$ -actinin inhibition of GRK5-mediated casein phosphorylation, but not  $\beta_2\text{AR}$  phosphorylation. Calmodulin, either 0.1  $\mu\text{M}$  (+) or 1.0  $\mu\text{M}$  (++), was incubated with 1.8  $\mu\text{M}$   $\alpha$ -actinin, 2 mM  $\text{CaCl}_2$  or 0.4  $\mu\text{M}$   $\alpha$ -actinin, 2 mM  $\text{CaCl}_2$  and casein (upper panel) or  $\beta_2\text{AR}$  (3 pmol) reconstituted in 80% PC vesicles (lower panel) and quantified as described in Section 2. Data are expressed as the percent of substrate phosphorylation in the absence of  $\alpha$ -actinin. The mean and S.E.M. of three independent experiments are shown for bars 1, 3, 4 and 5. A representative experiment is shown for bar 2.

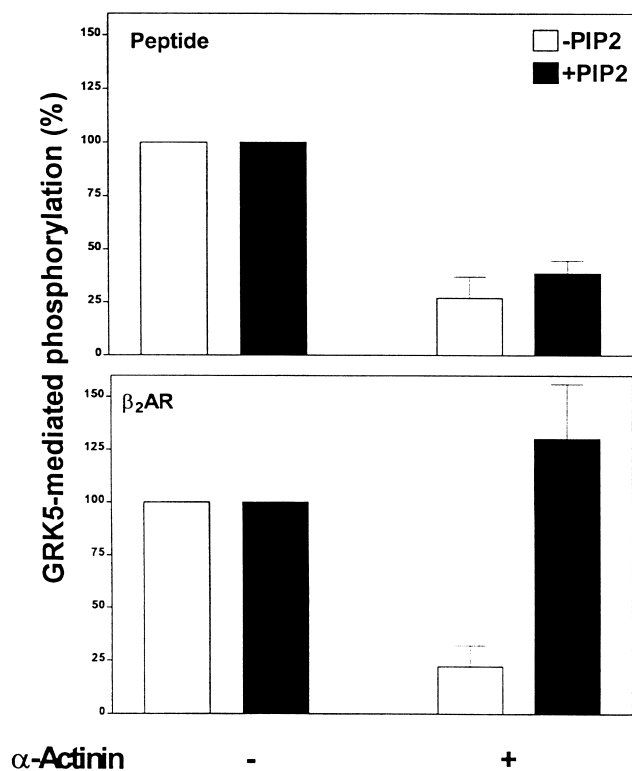


Fig. 4. PIP2 differentially affects  $\alpha$ -actinin-mediated inhibition of GRK5-mediated phosphorylation of peptide and  $\beta_2$ AR. GRK5 was incubated with peptide (upper panel) with or without 1.2  $\mu$ M  $\alpha$ -actinin or  $\beta_2$ ARs (lower panel) with or without 4.0  $\mu$ M  $\alpha$ -actinin in the presence of 100% PS (–PIP2) or 5% PIP2/95% PS (+PIP2) as indicated. GRK5-mediated phosphorylation was quantified as described in materials and methods. The data shown are the mean and S.E.M. of three independent experiments.

served with soluble substrates, addition of PIP2 to the  $\beta_2$ AR reconstitution vesicles relieved the inhibitory effects of  $\alpha$ -actinin (Fig. 4, lower panel, shaded columns). Thus calmodulin and PIP2 differentially regulate  $\alpha$ -actinin-mediated inhibition: calmodulin specifically relieves  $\alpha$ -actinin-mediated inhibition against soluble substrates while PIP2 exclusively relieves  $\alpha$ -actinin inhibition of membrane-bound substrates phosphorylation.

$\alpha$ -Actinin and spectrin contribute to the structural integrity of cells by crosslinking actin filaments and binding lipids which enables them to bridge membrane and cytoskeletal elements to produce large multi-protein complexes [9]. GRKs join a list of signal transducing proteins that bind  $\alpha$ -actinin and/or spectrin, including rabphilin 3A [22], PKN [23], PKC  $\epsilon$  [24] and NMDA receptors [19,25].

Contrary to  $\alpha$ -actinin and spectrin, calmodulin displaces numerous signaling molecules, including GRK5 [7,8] from binding to membranes and/or cytoskeletal proteins. One striking example is the NMDA receptor. Calcium-bound calmodulin competes with  $\alpha$ -actinin for binding to the NMDA receptor and is proposed to release the NMDA receptors from  $\alpha$ -actinin and cause a redistribution of receptors [19]. GRKs could also be localized by an analogous mechanism whereby  $\alpha$ -actinin anchors and inhibits GRKs and calcium-bound calmodulin modulates this association.

Phosphatidylinositols also play an important role in the regulation of  $\alpha$ -actinin and its binding proteins. PIP2 levels

increase the actin crosslinking ability of  $\alpha$ -actinin and co-localization of  $\alpha$ -actinin and PIP2 has been demonstrated in striated muscles cells [21]. Therefore, we propose that  $\alpha$ -actinin is involved in the cellular localization of GRKs and that PIP2 and calmodulin regulate the  $\alpha$ -actinin/GRK interaction in vivo as follows:  $\alpha$ -actinin inhibits GRKs under basal conditions. GPCR agonist stimulation, which increases intracellular PIP2 levels [26], would promote an interaction between  $\alpha$ -actinin and PIP2 that enables GRKs to phosphorylate membrane-incorporated receptor substrates. Conversely, when calcium-calmodulin levels increase, GRK5 is no longer inhibited by  $\alpha$ -actinin and does not interact with membranes, but rather preferentially phosphorylates soluble substrates.

In addition to elucidating a mechanism whereby not only GRK activity, but also GRK substrate specificity may be regulated, these data demonstrate that  $\alpha$ -actinin is a potent inhibitor of all GRKs. No highly specific inhibitors of GRKs have been identified. Expression of a peptide which inhibits GRK2/3 function in a genetic mouse model for human cardiac failure rescues the mouse from heart failure, suggesting a potential therapeutic role for inhibitors of GRK function [27]. Since  $\alpha$ -actinin potentially inhibits all GRKs, inhibitory peptides derived from  $\alpha$ -actinin represent a potential tool to directly inhibit the activity of the GRK family. Since GPCRs are involved in numerous human disease states and GRKs regulate GPCR function, development of a GRK inhibitor based on the  $\alpha$ -actinin sequence might have wide-ranging therapeutic possibilities.

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