

Phosducin inhibits receptor phosphorylation by the β -adrenergic receptor kinase in a PKA-regulated manner

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Abstract

Homologous or receptor-specific desensitization of β -adrenergic receptors is thought to be triggered by receptor phosphorylation mediated by the β -adrenergic receptor kinases (β ARK). Upon receptor activation, cytosolic β ARK translocates to the membrane, probably by binding to G-protein $\beta\gamma$ -subunits. Using the purified proteins reconstituted into phospholipid vesicles we show here that this binding process can be inhibited by phosducin, a cytosolic protein that has recently been described as a regulator of G-protein-mediated signalling. Phosducin appears to compete very effectively with β ARK for the G-protein $\beta\gamma$ -subunits. These inhibitory effects of phosducin on receptor phosphorylation are antagonized following phosphorylation of phosducin by protein kinase A. It is proposed that phosducin may act as a regulator of homologous β -adrenergic receptor desensitization.

Key words: β -Adrenergic receptor; β -Adrenergic receptor kinase; Phosducin; G-protein

1. Introduction

Signal transduction via G-protein-coupled receptors is regulated by multiple mechanisms, which have been investigated most thoroughly for the β -adrenergic receptor/ G_s /adenylyl cyclase [1] and the rhodopsin/ G_t /cyclicGMP phosphodiesterase [2] systems. Many of these mechanisms reduce receptor function in response to prolonged or repeated receptor stimulation, a phenomenon that is termed desensitization [3,4]. A major mechanism causing β -adrenergic receptor desensitization involves receptor phosphorylation catalyzed by the β -adrenergic receptor kinase (β ARK), followed by binding of β -arrestin to the phosphorylated receptors [5–8]. This mechanism, which can be mediated by two similar isoforms of both β ARK [9] and β -arrestin [10], appears to be the most rapid ($t_{1/2} < 15$ s) and quantitatively most important (> 50–70% loss of receptor function) mechanism causing desensitization of these and possibly other related receptors [11,12]. β ARK-catalyzed phosphorylation is specific for activated receptors because only the agonist-occupied receptors are a good substrate for β ARK [5]. More recently, a second agonist-dependent process enhancing β ARK-mediated phosphorylation has been identified: generation of activated G-proteins [13,14], so that their dissociated $\beta\gamma$ -subunits can serve as a membrane anchor for the cytosolic β ARK [15]. The

extent of this anchoring function of $\beta\gamma$ -subunits for β ARK has been shown to vary among different subtypes of β - and γ -subunits [16].

Protein kinase A can also phosphorylate β -adrenergic receptors [17], and this phosphorylation appears to cause per se desensitization, i.e. a reduction of the receptors' ability to activate G_s [11,17–21]. Most data suggest that the β ARK-mediated and the PKA-mediated pathways of β -adrenergic receptor desensitization are independent events [11,18,19]. However, in the case of olfactory receptor desensitization both PKA- and β ARK-like kinases appear to be required to effect desensitization [22]. One possible explanation for these data is that β ARK may be under the tonic control of an as yet unknown inhibitor, and that PKA may be capable of relieving this inhibition.

We have recently described that phosducin, a protein that had been thought to be specific for the retina and the developmentally related pineal gland [23,24], is a ubiquitous G-protein regulator [25]. Phosducin can bind to several different G-proteins, such as G_s , G_o , and G_i , and inhibits their GTPase- as well as their signalling functions [25]. Therefore, in the present study, we examined whether phosducin might also interfere with the enhancing effects of G-proteins on β ARK-mediated receptor phosphorylation using purified components reconstituted in phospholipid vesicles. Furthermore, since we had shown earlier that inhibition of G-protein function by phosducin was abolished following its phosphorylation by PKA [25], we investigated the effects of PKA-mediated phosphorylation of phosducin on β ARK translocation.

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Abbreviations: β ARK, β -adrenergic receptor kinase; PKA, protein kinase A; PKI, inhibitor of protein kinase A.

2. Materials and methods

2.1. Protein purification and reconstitution

Human β_2 -adrenergic receptors were expressed in Sf9 cells, and were purified by affinity chromatography [26]. Bovine β ARK-1 (which is the major isoform of β ARK for the phosphorylation of β_2 -adrenergic receptors; [9]) was expressed using the same system and purified [27]. Recombinant phosducin was purified from *E. coli* [25]. Purified β_2 -receptors and G_s , purified from rabbit liver [28] were reconstituted into phosphatidylcholine vesicles [29]. Urea-treated rod outer segments containing > 95% rhodopsin were prepared from bovine retina [30].

2.2. Receptor phosphorylation

20 μ l of vesicles containing 1 pmol of receptors and various amounts of G_s or resolved bovine brain G-protein $\beta\gamma$ -subunits were phosphorylated with 0.5 pmol purified β ARK (≈ 4 nM) in an assay volume of 120 μ l containing 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 8 mM $MgCl_2$, 1 mM dithiothreitol, 50 μ M [γ - ^{32}P]ATP (1 μ Ci/tube) during a 30 min incubation at 30°C (–)Isoproterenol and GTP were present at 10 μ M to activate receptors and G_s . Purified phosducin was present during this phosphorylation reaction in various concentrations. In some assays, phosducin was phosphorylated with purified PKA [31] prior to the β ARK assays; for those assays, control phosducin was incubated under the same conditions but without PKA. Following phosphorylation by PKA, 5 μ M protein kinase inhibitor (PKI, Sigma) was also included in order to prevent further phosphorylation of purified proteins. PKI did not inhibit specific receptor phosphorylation by β ARK (not shown). The receptor phosphorylation by β ARK was stopped with 1 ml of ice-cold incubation buffer and centrifugation at 300,000 $\times g$ for 30 min, and the pellets were subjected to SDS-PAGE. The phosphorylated bands were visualized by autoradiography, and the radioactivity incorporated into the receptor band was quantitated by Cerenkov counting.

Rod outer segments (≈ 25 pmol rhodopsin/60 μ l) were phosphorylated in a similar manner, except that bright white light was used for activation, and the incubation time was 10 min. Assay conditions for phosphorylation of the soluble β ARK-substrate peptide RRRAEAASAAA (2 mM, [32]) by purified recombinant β ARK (15 nM) were similar to those used for receptor vesicles, except that the assay volume was 30 μ l and BSA was added to 0.5 mg/ml. The incubations were stopped by spotting onto Whatman P-81 phosphocellulose paper and immersion in phosphoric acid [32].

2.3. Data analysis

Concentration-response curves were analyzed by non-linear curve-fitting in order to determine IC_{50} values as described [33]. Unless stated otherwise all data are from at least 3 independent experiments.

3. Results

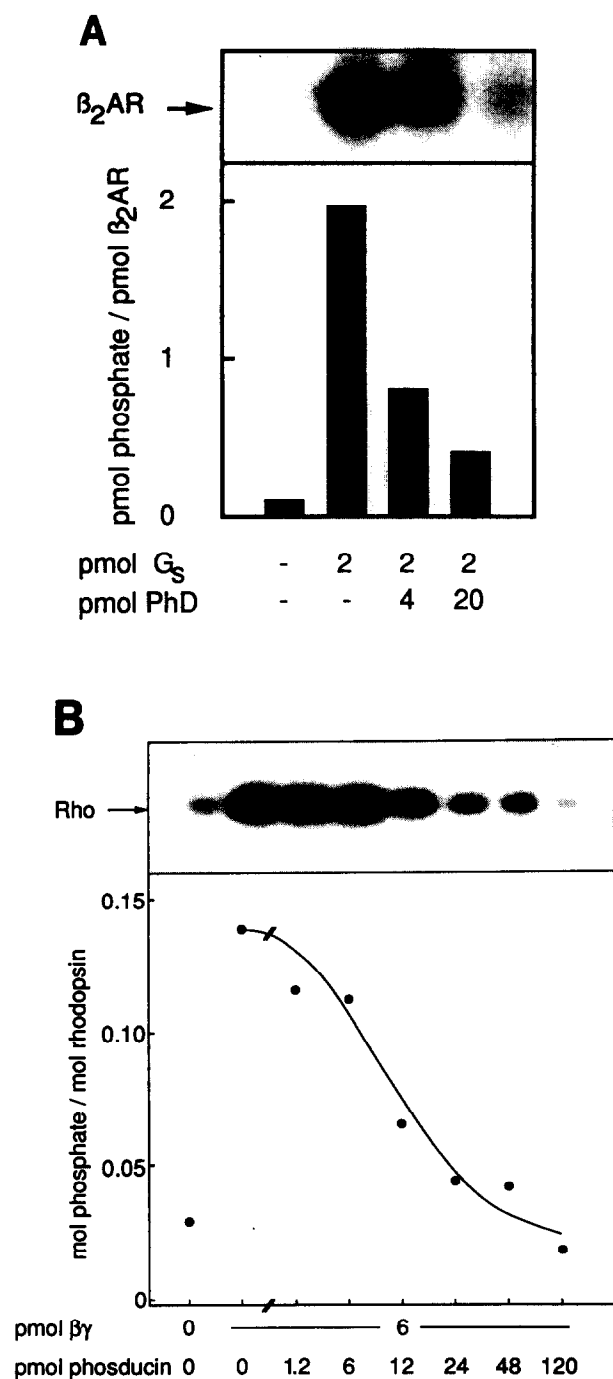
In a system consisting of the purified components, β_2 -adrenergic receptors and G_s reconstituted into phospholipid vesicles, addition of phosducin markedly inhibited β ARK-catalyzed receptor phosphorylation (Fig. 1A). The presence of G_s increased phosphorylation more than 10-fold to 2 mol phosphate/mol receptor. Similar results were obtained with resolved G-protein $\beta\gamma$ -subunits instead of G_s (not shown). Phosducin completely negated this enhancing effect of G_s , with half-maximal inhibition achieved at a ratio of phosducin: G_s of below 2. Considering that phosducin is a soluble protein whereas G_s was reconstituted into the vesicles, this shows a remarkable potency of phosducin and suggests that inhibition may be caused by formation of a 1:1 phosducin-G-protein complex. Similar effects were observed when the extent of phosphorylation was increased

(6 mol phosphate/mol receptor), either by extended incubation times or by the use of higher β ARK concentrations (not shown). G-proteins were required for the inhibition, since phosducin had no effect on the residual phosphorylation occurring in the absence of G-proteins (not shown).

β ARK can also phosphorylate rhodopsin in a light-dependent manner, albeit to a much lower degree than the specific rhodopsin kinase [34]. Phosducin was an equally potent inhibitor in this system (Fig. 1B). In these experiments, we added G-protein $\beta\gamma$ -subunits to rod outer segments, which contain > 95% rhodopsin. The resolved $\beta\gamma$ -subunits caused an > 5-fold enhancement in phosphorylation (Fig. 1B), and similar results were obtained with trimeric G_s (not shown). Again, this enhancement was completely reversed by phosducin. Half-maximal inhibition occurred at ≈ 155 nM phosducin; this value is ≈ 5 times higher than the one that can be estimated from Fig. 1A (< 33 nM), in line with the 6-fold higher $\beta\gamma$ -subunit concentration which was used in these experiments, and corresponds to a molar ratio (phosducin: $\beta\gamma$ -subunit) of 1.5. When a short soluble peptide substrate [32] for β ARK was used (Fig. 1C), there was no inhibition even by high concentrations of phosducin (600 nM, 40-fold molar excess over β ARK), suggesting that phosducin does not directly inhibit β ARK.

Phosducin inhibited the association of β ARK with the phospholipid vesicles in the presence of G-protein $\beta\gamma$ -subunits (Fig. 2A) or trimeric G_s (not shown). The decrease of vesicle-associated β ARK as demonstrated by Coomassie blue staining of the vesicle components was paralleled by decreased vesicular β ARK-activity as measured by β ARK-autophosphorylation (Fig. 2A) or β_2 -receptor phosphorylation (not shown). In the absence of G-proteins or their $\beta\gamma$ -subunits there were no detectable amounts of β ARK associated with the vesicles as shown by Coomassie blue staining. This extent was not altered by the presence of phosducin (not shown). Thus, phosducin could displace β ARK from $\beta\gamma$ -subunit-containing vesicles. In a reciprocal manner, β ARK could displace phosducin from the same vesicles (Fig. 2B), but a higher stoichiometry of β ARK vs. phosducin was required. This suggests that β ARK and phosducin compete for G-protein- $\beta\gamma$ -subunits as membrane anchors, with phosducin being the more potent competitor.

We have shown earlier [25] that the inhibitor effects of phosducin on G-protein-mediated signalling were almost completely lost after phosphorylation of phosducin by protein kinase A (PKA), which occurs as serine-73 [35]. Similarly, the inhibitory effects of phosducin on G-protein-enhanced β_2 -receptor phosphorylation were markedly impaired by PKA-mediated phosphorylation of phosducin (Fig. 3). PKA-phosphorylated phosducin was more than 10-fold less potent than non-phosphorylated (but similarly treated) phosducin.



The IC₅₀ value for control phosducin in this experiment (≈ 80 nM) was higher than the one that can be estimated from Fig. 1A (< 33 nM). This may be due to the higher βARK concentration used in the experiment shown in Fig. 3. Dependence of the IC₅₀ value both on the amounts of G-protein βγ-subunits (Fig. 1A vs. Fig. 1B) and the concentration of βARK (Fig. 1A vs. Fig. 3) is consistent with the notion of competition of βARK and phosducin for the βγ-subunits as membrane anchors.

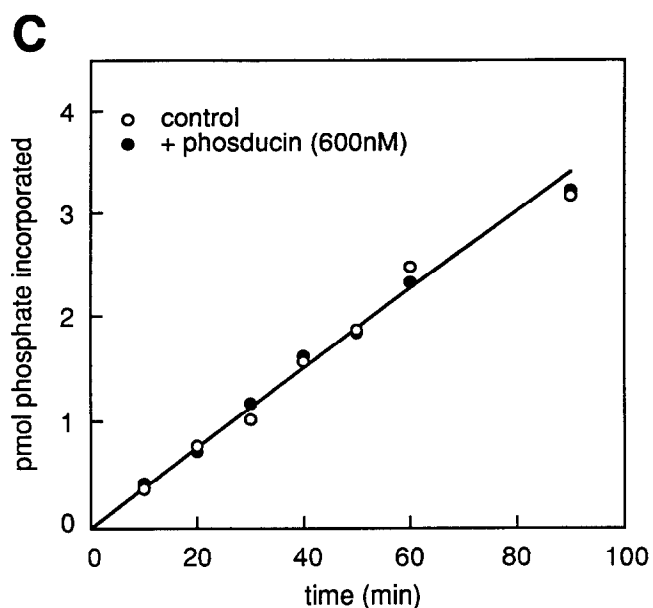


Fig. 1. Effects of phosducin on βARK-catalyzed phosphorylation. (A) Inhibition of the phosphorylation of purified β₂-adrenergic receptors reconstituted with or without purified G_s into phospholipid vesicles. Top panel: autoradiogram of phosphorylated receptors after electrophoresis on an SDS-polyacrylamide gel. Bottom panel: quantitation of phosphate incorporation into receptors. 20 μl of vesicles containing 1 pmol of receptors and 0 or 2 pmol of G_s (as indicated) were phosphorylated with 0.5 pmol purified βARK (≈ 4 nM) in the presence of 0, 4 or 20 pmol purified phosducin (≈ 33 and ≈ 167 nM) in an assay volume of 120 μl. (B) Inhibition of the phosphorylation of rhodopsin in rod outer segments in the presence of G-protein βγ-subunits purified from bovine brain. Top panel: autoradiogram of phosphorylated rhodopsin after electrophoresis on an SDS-polyacrylamide gel. Bottom panel: quantitation of phosphate incorporation into rhodopsin. Urea-treated rod outer segments (≈ 25 pmol rhodopsin) were phosphorylated in the presence of resolved bovine brain G-protein βγ-subunits (0 or 6 pmol) in an assay volume of 60 μl-assay. Curve-fitting [33] gave an IC₅₀ value of 9.3 pmol phosducin (≈ 155 nM). (C) Unaltered phosphorylation of a peptide substrate by βARK in the presence of phosducin. The synthetic peptide RRRAEAASAAA (2 mM) was phosphorylated by purified recombinant βARK (15 nM) in the absence (○) or presence (●) of purified recombinant phosducin (600 nM), and phosphate incorporation was determined after the indicated incubation times. Data in all panels are from single experiments representative of at least three similar experiments.

4. Discussion

The recent discovery of a role for G-proteins in the phosphorylation and, hence, presumably the desensitization of β-adrenergic as well as muscarinic receptors has added a new level of complexity to the mechanisms regulating these receptors. We now find that this regulatory role of G-proteins may itself be subject to regulation by phosducin. In its non-phosphorylated state phosducin can bind to G-proteins; this binding appears to occur primarily to the βγ-subunits of G-proteins [23,25]. When phosducin is bound to G-proteins, it inhibits binding of βARK to the G-protein βγ-subunits and thereby reduces βARK-mediated receptor phosphorylation. Following

phosphorylation of phosducin by PKA phosducin appears to dissociate from the G-proteins and to permit binding of β ARK to the $\beta\gamma$ -subunits of the activated G-proteins, and thus allow receptor desensitization. In agreement with earlier data, the interaction of phosducin with G-protein $\beta\gamma$ -subunits seems to depend on the phosphorylation status of phosducin.

These hypotheses suggest that β -adrenergic receptors, as well as possibly other related receptors, are embedded in a complex network regulating their function, and that phosducin may be a component of this network. It may have dual functions in this receptor system: in its non-phosphorylated state it may dampen G-protein-mediated signalling [25] but may at the same time reduce β ARK-mediated receptor desensitization. In its phosphorylated state, it might dissociate from the G-proteins and thereby permit receptor desensitization. Further experiments will have to address the question whether intracellular phosducin levels are sufficient to exert a similar inhibition of receptor phosphorylation under physiological conditions.

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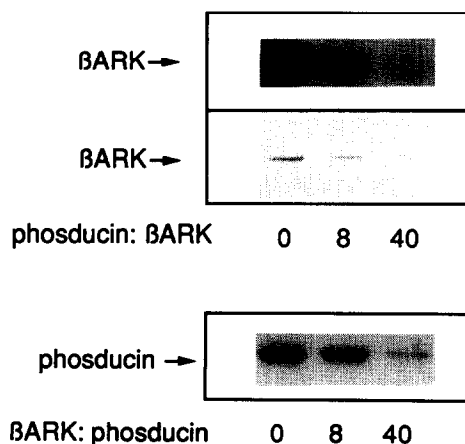


Fig. 2. Competition of β ARK and phosducin for G-protein $\beta\gamma$ -subunits in phospholipid vesicles. (A) Phosducin inhibits the association of β ARK with $\beta\gamma$ -containing vesicles. (B) β ARK inhibits the association of phosducin with $\beta\gamma$ -containing vesicles. Shown are vesicle-associated β ARK (A, top panel: autoradiography of β ARK; bottom panel: Coomassie-stain of β ARK) or phosducin (B, Coomassie stain) after incubation of β_2 -receptor (1 pmol)- and G-protein $\beta\gamma$ -subunit (8 pmol)-containing phospholipid vesicles with a constant amount of β ARK and varying amounts of phosducin at ratios phosducin: β ARK of 0, 8 and 40 (A) or a constant amount of phosducin and varying amounts of β ARK giving β ARK:phosducin ratios of 0, 8, 40. (B). The assay contained 50 μ M [γ - 32 P]ATP to allow visualization of β ARK by autoradiography, and were carried out as described under methods. Vesicle-associated proteins were identified by SDS-PAGE of the vesicle pellet and subsequent autoradiography or Coomassie-staining of the gels.

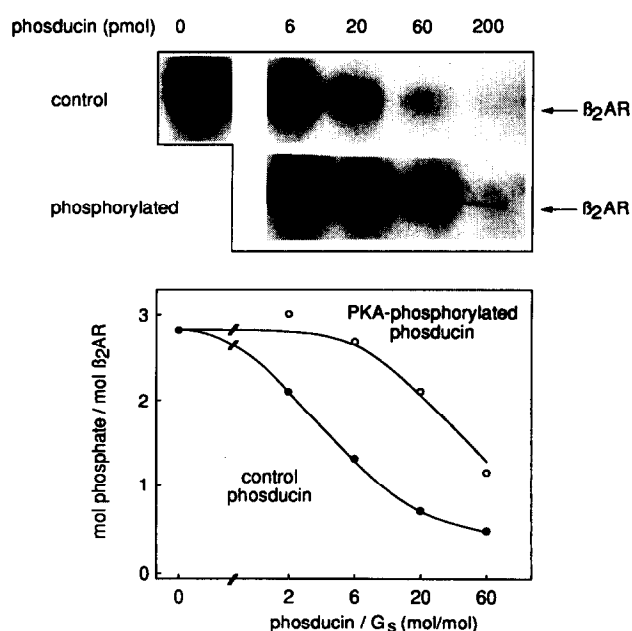


Fig. 3. PKA-catalyzed phosphorylation of phosducin antagonizes its inhibitory effects on G-protein-dependent β_2 -adrenergic receptor phosphorylation. Top panel: autoradiogram of phosphorylated receptors after electrophoresis on an SDS-polyacrylamide gel. Bottom panel: quantitation of phosphate incorporation into receptors. β_2 -adrenergic receptors (0.5 pmol/tube) were reconstituted with or without G_s (3 pmol/tube) and phosphorylated by β ARK (5 pmol/tube) in the absence or presence of varying concentrations of phosducin. Phosducin was phosphorylated by PKA (or treated similarly without PKA: control phosducin) prior to the assay. Further PKA-mediated phosphorylation was then blocked by the addition of 5 μ M PKI. Phosducin was present at 0, 6, 20, 60 or 200 pmol/tube, giving molar phosducin/ G_s ratios of 0, 2, 6, 20 and 60. IC_{50} values calculated by curve-fitting [33] were $\approx$ 80 nM (control phosducin) and $\approx$ 1000 nM (PKA-phosphorylated phosducin).

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