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The basal subcellular distribution of β-adrenergic receptor kinase is independent of G-protein βγ subunits

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Abstract β-adrenergic receptor kinase (βARK-1 or GRK2) is a key regulatory protein involved in the regulation of G-protein-coupled receptors which associates with microsomal and plasma membranes. βγ subunits of G-proteins have been suggested to mediate agonist-dependent membrane translocation of βARK, but their possible role in maintaining the complex subcellular distribution of the kinase is not known. In this study we show that lovastatin-mediated inhibition of Gγ subunits isoprenylation in HEK-293 cells stably transfected with βARK1 leads to a significant release of Gβ subunits to the cytosol without causing changes in total particulate βARK or in the association of this kinase to plasma or microsomal membrane fractions. In addition, transient overexpression of mutant forms of Gγ unable to become isoprenylated resulted in a marked sequestration of Gβ to the soluble compartment, but caused no rearrangement in the distribution of cotransfected βARK. These results indicate that anchoring of βARK to cellular membranes under basal conditions is independent of the availability of heterotrimeric G-protein subunits.

Key words: G-protein-coupled receptor kinase; G-protein-coupled receptor; β-adrenergic receptor kinase; Isoprenylation; Kinase anchor; G-protein

1. Introduction

β-adrenergic receptor kinase (βARK)[1] is a ubiquitous serine-threonine kinase able to phosphorylate specifically the agonist-bound form of several G-protein-coupled receptors (GPCR). βARK belongs to a family of kinases termed GRKs (for G-protein-coupled receptor kinases) that have been shown to play a key role in the processes of desensitization of seven transmembrane-spanning receptors [1–4]. βARK-mediated phosphorylation triggers the association to the receptor of a protein named β-arrestin, which inhibits the receptor-mediated activation of G-proteins thus bringing about the termination of the response [3,4]. Recent studies have also involved βARK and β-arrestin proteins in the subsequent process of receptor internalization [5,6].

βARK was initially described as a cytosolic enzyme able to transiently translocate to the plasma membrane in response to agonist stimulation [7,8]. Based on in vitro observations, βγ subunits of G-proteins have been suggested to play a role in βARK translocation to the cell surface upon receptor activation [2,4]. In addition, we have recently reported that a significant amount of the kinase can be found in association with intracellular microsomal membranes under basal, unstimulated conditions by means of a high-affinity ($K_d = 20$ nM) binding to an unidentified microsomal anchoring protein [9]. βγ subunits have been shown to both interact with βARK [10,11] and enhance several-fold the catalytic activity of βARK towards different G-protein-coupled receptors [10,12]. Recent studies have mapped the βγ-binding domain to a C-terminal region corresponding to amino acids 546–670 of the bovine βARK1 [13]. This region is also present in the βARK2 (GRK3) isoform. The fact that the highest sequence heterogeneity between GRK2 and GRK3 lies in their C-terminus may account for different binding affinities to Gβγ subunits [14]. The Gβγ-binding region partially overlaps with a pleckstrin homology (PH) domain (amino acids 553–651) [15] which has been implicated in the association of the kinase to different kinds of lipids which may contribute to the regulation of βARK localization and activity [16,17]. The PH domain of βARK could therefore represent a way of anchoring the kinase to intracellular membranes through protein–lipid or protein–protein interactions.

Interestingly, it has been shown that although βγ dimers are the units responsible for βARK activation, binding of this kinase to Gβγ is achieved equally well when the whole G-protein heterotrimer is used as a ligand [11], thus suggesting that G-proteins may play a role as general kinase anchors in different cellular membranes and experimental conditions. However, the fact that the microsomal-anchoring domain has been mapped to an N-terminal region of βARK distinct from the βγ-binding segment suggests that proteins different from βγ subunits of G-proteins could also be involved in kinase attachment and in modulating βARK subcellular distribution [9,18]. In this context, and considering that in situ studies have not been performed with full-length wild-type kinase regarding its association to G-proteins under basal conditions, we have investigated the effects of changes in membrane-bound Gβγ subunits on the subcellular pattern of βARK. Our results lend further support to the notion that the association of the kinase to different cellular membranes under basal situations is accomplished via additional anchoring mechanisms.

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Abbreviations: GPCR, G-protein-coupled receptor(s); βARK, β-adrenergic receptor kinase; G-protein, guanine nucleotide-binding protein; GRK, G-protein-coupled receptor kinase; Gβ, G-protein β subunit(s); Gγ, G-protein γ subunit(s); SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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2. Materials and methods

2.1. Materials

Human embryonic kidney EBNA-293 cells were obtained from Invitrogen Corporation. Lovastatin was kindly provided by Merck Sharp and Dohme (Rahway, NJ). Antibiotics were purchased from Calbiochem, sera from Gibco and culture plates from Costar. Antibodies against G-protein β and γ subunits were obtained from Santa Cruz Biotechnology. Electrophoresis reagents were from Bio-Rad. All other reagents were of the highest purity available.

2.2. Cell culture, transfection and treatments

HEK-293 cells stably overexpressing bovine βARK1 were generated as described [6]. They were grown in monolayers over P-100 dishes previously coated with 10 μg/ml poly-L-lysine in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum and antibiotics (gentamicin 50 μg/ml, Streptomycin 0.01% and Penicillin G 0.063%) together with hygromycin (0.25 mg/ml) and geneticin (0.2 mg/ml). Cells were grown to confluence in a water-saturated atmosphere at 37°C and 7% CO_2. For cell treatment, lovastatin was converted to the lactone sodium salt as described [19], diluted to 10 mM and kept frozen at −20°C until used. Cells were incubated for 48 h in the presence of 15 μM lovastatin or vehicle as a control.

For transient expression experiments, HEK-293 cells were transfected with 2.5 μg/dish of pREP4-βARK, that contained the full-length form of bovine βARK1 cDNA, alone or in combination with diverse constructs of G-protein subunits, using the calcium phosphate precipitation method [20]. Mutant forms of $G\gamma_1$ or $G\gamma_2$ subunits that are unable to become isoprenylated (pCMV-γ_1* or pcDM8.1-γ_2*, 18 μg/dish) were transiently overexpressed to specifically sequester endogenous Gβ subunits to the cell cytosol [21]. In some experiments wild-type $G\beta_2$ and $G\gamma_2$ subunits (pcDNAI-$G\beta_2$ and pcDM8.1-$G\gamma_2$, 9 μg/dish of each construct) were also used [22,23]. All G-protein constructs were kindly donated by Dr. Melvin I. Simon (Caltech, Pasadena). The amount of total DNA per dish was adjusted with empty vector. Transfection efficiency was 15–20% as assessed by indirect immunofluorescence using anti-βARK antibodies [18]. Cells were harvested 36 h after transfection and processed as described below.

2.3. Subcellular fractionation

Cell fractionation was performed by a modification of the method described in [18]. Briefly, cells were harvested and washed extensively with phosphate-buffered saline. The resulting cell pellet was resuspended in 300 μl of lysis buffer (5 mM Tris-HCl (pH 7.5), 2 mM EDTA, aprotinin 8 μg/ml, phenylmethylsulphonyl fluoride 100 μM, and 100 μg/ml each of soybean trypsin inhibitor, bacitracin and benzamidine) per dish and homogenized by two 30-s strokes in a Polytron PT 3000 apparatus. Unbroken cells and nuclei were sedimented by centrifugation (800×*g*, 4 min) and particulate plus soluble fractions were obtained by a single high-speed centrifugation step (230 000×*g*, 40 min) in a Beckman TL100.1 ultracentrifuge. In some experiments, a crude plasma membrane pellet was obtained by centrifugation at 12 000×*g* for 20 min at 4°C. A subsequent centrifugation step at 230 000×*g* for 45 min separated the crude microsomal membrane preparation and the soluble cytosolic fraction. To extract peripherally associated proteins, membrane fractions were resuspended and incubated in 200 mM NaCl for 30 min at 4°C and recentrifuged in the same conditions [18].

2.4. Immunoblotting

Western blots were performed as described [9]. Briefly, equal amounts of proteins from each cellular fraction were resolved by electrophoresis in 11% SDS-PAGE and blotted onto nitrocellulose filters in a Bio-Rad wet chamber electrotransfer apparatus following manufacturer's instructions. The upper part of the filter (molecular mass range 66–97 kDa) was then incubated with a polyclonal antiserum against recombinant βARK (Ab9, 1:1000, kindly provided by Dr. J.L. Benovic, Thomas Jefferson Cancer Institute, Philadelphia, PA) or a purified polyclonal antiserum raised against a GST-βARK(50–145) fusion protein (Ab-FP1, 5 μg/ml). The lower part of the filter (molecular mass range 21–45 kDa) was incubated with M14 anti-Gβ common polyclonal antibody (5 μg/ml, Santa Cruz Biotechnology). Blots were developed by a chemiluminiscent method (ECL, Amersham) and bands corresponding to βARK (80 kDa) or Gβ (36–37 kDa) were subjected to quantitation by densitometric analysis in a Molecular Dynamics Laser densitometer.

3. Results and discussion

With the aim to investigate the role played by heterotrimeric G-proteins in maintaining the complex subcellular distribution of βARK in situ, we took advantage of the well-documented fact that the anchoring of Gγ subunits to membranes is achieved by means of its isoprenyl moiety [24,25] to modify

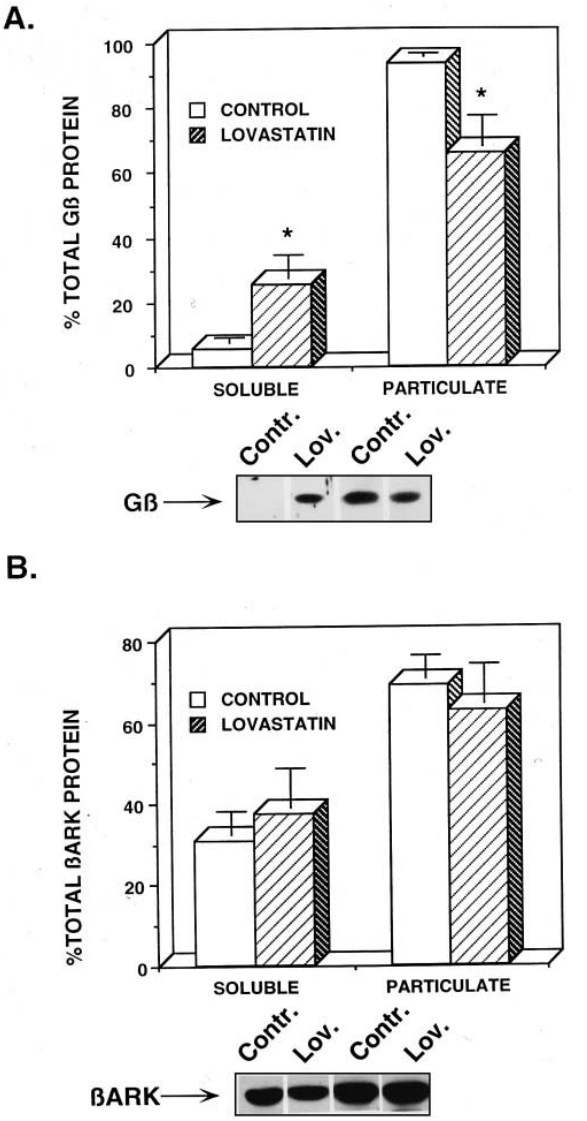

Fig. 1. Effect of lovastatin treatment on the subcellular distribution of βARK and G-protein β subunits. HEK-293 cells stably transfected with βARK1 were treated or not with the active form of lovastatin (15 μM) for 48 h. After cell lysis and centrifugation, the same amount of protein (60 μg) from particulate and soluble fractions (prepared as described in Section 2) was resolved by 11% SDS-PAGE followed by blotting onto nitrocellulose membranes. The lower part of the blot was incubated with M14 anti-Gβ antibody (5 μg/ml, panel A) and the upper part of the blot with anti-βARK Ab9 antibody (1:1000, panel B). The blots were developed and quantified by densitometric analysis. Data were referred to total cellular βARK or Gβ protein and results from 4–6 independent experiments were subjected to statistical evaluation using unpaired Student's *t* test. *$P < 0.05$ with respect to control cells.

the subcellular distribution of Gβγ dimers in cultured cells. Lovastatin (a competitive inhibitor of hydroxymethylglutaryl-CoA reductase, a key enzyme in isoprenoid metabolism) has been shown to promote an important solubilization of heterotrimeric G-proteins in several systems [26–28]. In this line, we tested the effects of lovastatin treatment of HEK-293 cells stably transfected with βARK on the membrane association of both endogenous Gβ-protein and the kinase by Western blot analysis of soluble and particulate fractions. βARK overexpression does not lead to changes in the subcellular localization of the kinase compared to wild-type cells (data not shown). Fig. 1A shows that lovastatin caused a clear redistribution of Gβ subunits from the particulate to the soluble pool, where the percentage of total Gβ-protein found increased from 5 ± 2% in control cells to 25 ± 2% in treated cells. The corresponding decrease in the amount of G-proteins present in the particulate fraction (Fig. 1A) failed to provoke any significant impairment in the membrane association of βARK, which displays a similar subcellular pattern of immunoreactivity in control and treated cells (Fig. 1B).

In a previous in vitro study [9] we have reported that recombinant βARK is able to interact with a protein component of microsomal membrane preparations via a new targeting domain located in the N-terminal region of the kinase, thus suggesting the involvement of anchoring proteins different from Gβγ subunits. To explore the possibility of a differential effect of lovastatin treatment on Gβγ presence in the diverse cellular membranes, or a distinct involvement of Gβγ subunits in kinase anchoring to the various cellular locations, a more detailed study was performed. Fig. 2 shows the amount of Gβ subunits and βARK protein present in different cellular fractions (cytosol, plasma membrane and microsomal membranes) in control and treated cells as visualized by Western blot. A marked change in the amount of Gβ subunits associated to both plasma and microsomal membranes is observed (compare lanes − to + lovastatin under PM and MM) whereas an increase takes place in the soluble cytosolic compartment (that contained 48 ± 3% of total Gβ in treated cells, mean ± SEM of three experiments). On the other hand, βARK immunoreactivity in the different compartments revealed an unaltered distribution and, when normalized for total cellular protein, the distribution of kinase in the different subcellular fractions was in agreement with previously published data [18]. Our results indicate that important changes in the different membrane pools of Gβ subunits do not lead to a rearrangement of βARK subcellular distribution in situ. In particular, the data regarding microsomal fractions are in agreement with previous in vitro studies showing that depletion of approximately half the population of endogenous heterotrimeric G-proteins from crude microsomal membranes by alkaline extraction did not affect the ability of recombinant βARK to associate to membranes [9], thus further arguing in favor of the implication of a new anchoring component.

In order to rule out the possibility that lovastatin treatment could be exerting indirect, compensatory effects on βARK membrane association by means of its actions on other isoprenylated cellular proteins, we used a more direct strategy to modify the ratio of soluble to particulated Gβ subunits in cells. In this experimental approach, we cotransfected HEK-293 cells with βARK1 constructs and mutant forms of Gγ

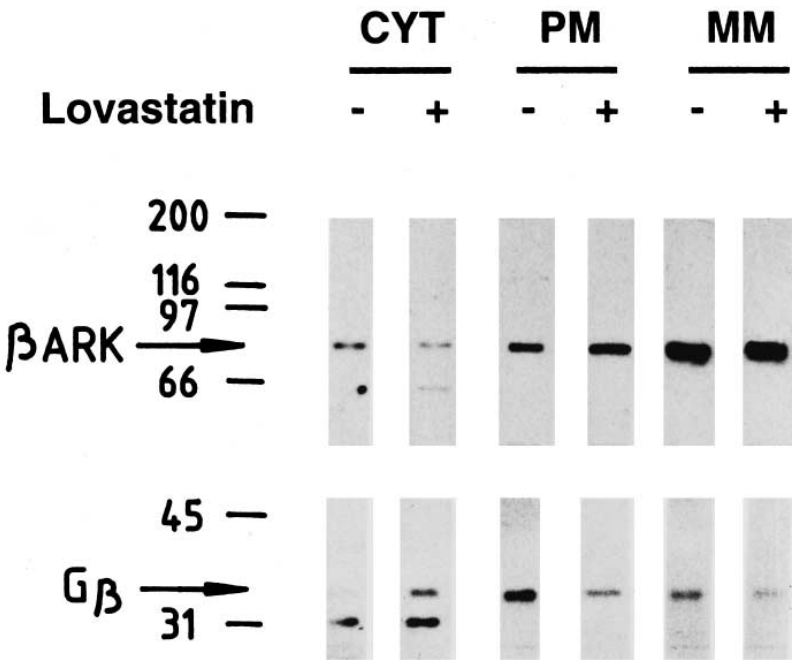

Fig. 2. Effect of lovastatin treatment on Gβ subunits and βARK distribution in different cellular fractions. Subcellular fractions of HEK-293 cells stably expressing bovine βARK1 were obtained as specified in Section 2 from cells grown to confluence treated (+) or not (−) for 48 h with 15 μM lovastatin. For βARK analysis, equal amounts (20 μg) of proteins from the cytosolic fraction (CYT) or extracted by 200 mM NaCl treatment from either plasma membrane (PM) or microsomal membranes (MM) fractions were resolved by 11% SDS-PAGE. Immunoblot analysis was performed using an affinity-purified anti-βARK antibody (anti-GST-βARK(50–145), 5 μg/ml). For Gβ quantitation, equal amounts (20 μg) of cytosol (CYT) or plasma membrane (PM) or microsomal membrane (MM) fractions containing the proteins that remained attached to the membranes after salt extraction, were resolved, blotted and analyzed with anti-Gβcommon M14 antibody (5 μg/ml). The migration of βARK and Gβ is indicated by arrows and that of the molecular mass standards is shown in the left margin of the figure. Results are representative of three independent experiments.

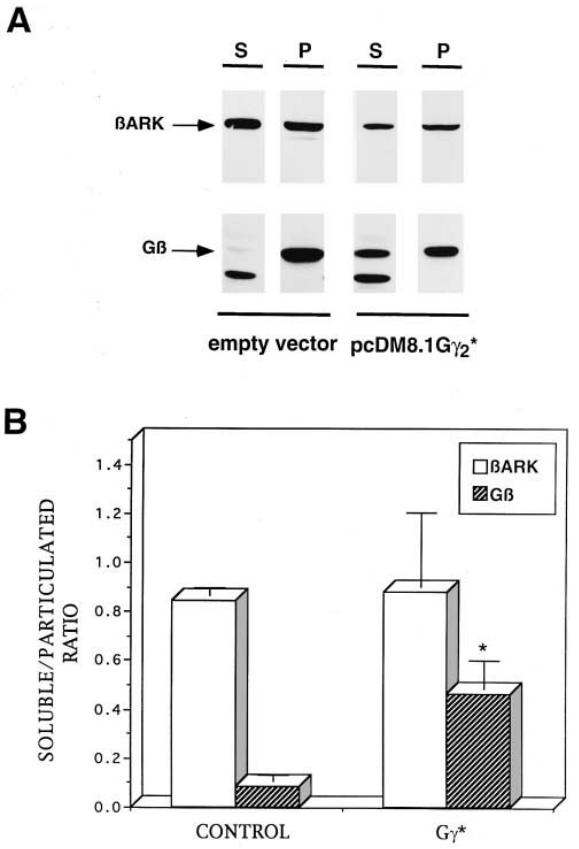

Fig. 3. Effect of overexpression of isoprenylation-deficient Gγ subunit mutants (Gγ*) on the subcellular distribution of endogenous Gβ subunits and cotransfected βARK1 in HEK-293 cells. HEK-293 cells were transiently co-transfected with pREP4-βARK1 and either pCMV-γ_1*, pcDM8.1-γ_2* or empty vector as a control. Cells were processed as described in Fig. 1. A representative blot is shown in panel A. The migration of βARK and Gβ-proteins is indicated by arrows. Panel B displays ratios of soluble to particulate protein as calculated from densitometric analysis of the blots. Data are means ± SEM of three independent experiments. Significant differences were found for Gβ ratios ($^*P < 0.02$) but not for those of βARK ($P = 0.924$).

subunits unable to become isoprenylated [21]. A marked transfer of Gβ subunits from particulate (P) to soluble (S) fractions is visualized by Western blot analysis in cells co-transfected with $G\gamma_2$* (Fig. 3A) or $G\gamma_1$* (not shown) mutant constructs and βARK. A significant increase in the soluble versus particulate Gβ ratio in the cells expressing γ* subunit mutants is detected by densitometric analysis of the immunoblots (Fig. 3B, hatched bars). Again, such variation in Gβ subunits caused no significant change in the ratio of soluble to particulated βARK (Fig. 3A and open bars in Fig. 3B). Interestingly, when wild-type β_2 and γ_2 constructs were co-transfected along with the kinase, the ratio of soluble versus particulated kinase decreased from 0.85 ± 0.03 in control cells to 0.47 ± 0.04 in Gβγ-transfected (means ± SEM of three experiments), thus indicating an enhanced membrane association of the kinase probably due to an increased availability of free Gβγ dimers in the particulate pool. Since this situation would reflect the release of Gβγ that takes place after receptor stimulation, our data suggest that, although not readily involved in the association of βARK to cellular membranes under basal conditions, Gβγ subunits may play a role in agonist-mediated targeting of the kinase to the membrane in intact cells. It is also worth noting that a slight decrease in βARK levels is observed in the cells expressing γ* subunit mutants (Fig. 3A), thus raising the possibility that kinase expression or stability could be modulated in such experimental conditions.

The results described in the present study provide evidence to support new types of cellular interactions for βARK, independent from its association to G-protein βγ subunits. Although βγ binding to the C-terminal region of βARK is well documented [29–31], the relevance of the association of the full-length kinase to Gβγ in situ remained to be established. The fact that additional ligands for βARK have been recently described, including several types of phospholipids [16,32] and a microsomal protein component [9,18] suggest that complex mechanisms operate to govern the intracellular localization of βARK. Our results strongly suggest that the association of the kinase to intracellular particulate pools under basal conditions is not predominantly accomplished by attachment to heterotrimeric G-protein subunits. It is possible that interactions with lipids may play a role in βARK anchoring to cellular particulate fractions. However, it should be noted that anchoring of the kinase to microsomal membranes in vitro is both heat and protease sensitive, and requires a N-terminal domain of the kinase that does not include the reported lipid-binding PH domain [9]. Therefore, the association of βARK with specific membrane-localized anchoring proteins (as described for other protein kinases, reviewed in [33]) may represent an essential mechanism for regulating the localization and possibly the kinase activity of this key regulatory protein [9,17]. Further experimental efforts will be required to identify such anchoring protein(s) and its biological role in the complex regulation of βARK localization and function.

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