Protein kinase cross-talk: Membrane targeting of the β-adrenergic receptor kinase by protein kinase C

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ABSTRACT The β-adrenergic receptor kinase (βARK) is the prototypical member of the family of cytosolic kinases that phosphorylate guanine nucleotide binding-protein-coupled receptors and thereby trigger uncoupling between receptors and guanine nucleotide binding proteins. Herein we show that this kinase is subject to phosphorylation and regulation by protein kinase C (PKC). In cell lines stably expressing αβ-receptor kinases, activation of these receptors by epinephrine resulted in an activation of cytosolic βARK. Similar data were obtained in 293 cells transiently coexpressing αβ-receptor kinases and βARK-1. Direct activation of PKC with phorbol esters in these cells caused not only an activation of cytosolic βARK-1 but also a translocation of βARK immunoreactivity from the cytosol to the membrane fraction. A PKC preparation purified from rat brain phosphorylated purified recombinant βARK-1 to a stoichiometry of 0.86 phosphate per βARK-1. This phosphorylation resulted in an increased activity of βARK-1 when membrane-bound rhodopsin served as its substrate but in no increase of its activity toward a soluble peptide substrate. The site of phosphorylation was mapped to the C terminus of βARK-1. We conclude that PKC activates βARK by enhancing its translocation to the plasma membrane.

Signaling via guanine nucleotide binding (G) protein-coupled receptors is initiated by binding of agonist ligands and is mediated by the activation of G proteins, which involves binding of GTP by the α subunit and its dissociation from the βγ-subunit complex (1, 2). Both the α subunit and the βγ-subunit complex can then regulate effector molecules and thereby produce intracellular signals (3, 4).

This signaling pathway is subject to a variety of regulatory mechanisms that alter the expression and the function of the proteins involved. One of the key regulatory processes is the desensitization of such receptors in response to prolonged or repeated agonist exposure. Various mechanisms contribute to receptor desensitization (5, 6). The most rapid and probably quantitatively the most important one (7, 8) is triggered by phosphorylation of the receptors by members of the family of G-protein-coupled receptor kinases. Of the six members of this family cloned to date (9), the β-adrenergic receptor kinase-1 (βARK-1; ref. 10) has been studied as the prototypical member that mediates receptor desensitization. Desensitization by this kinase occurs as a two-step process: (i) The kinase translocates from the cytosol to the plasma membrane (11) and phosphorylates several serine and threonine residues in the cytoplasmic portions of the receptors. (ii) Members of another family of cytosolic proteins, the arrestins, bind to the phosphorylated receptors and thereby impair the receptor–G-protein interaction (12–14). Much interest has recently been concerned with the mode of translocation of βARK to the plasma membrane. Three anchoring points have been identified that are used for membrane attachment: the agonist-occupied receptor (15), the G-protein βγ subunits (16–18), and phosphatidylinositol bisphosphate (19). The agonist-activated receptors and the G-protein βγ subunits have been reported to cause activation of the kinase (15, 20), but no such activation has been observed by other authors (19) for the G-protein βγ subunits and for phosphatidylinositol bisphosphate. Thus, βARK appears to be regulated essentially by the availability of the substrate (since only the agonist-occupied form of receptors is a substrate) and of the membrane anchors. In many other kinases, there are multiple mechanisms that regulate the kinase activity, for example, via phosphorylation of the kinase, but no such mechanisms have so far been identified for βARK.

In addition to βARK, two other kinases have been found to be capable of phosphorylating β2-adrenergic receptors, i.e., protein kinase A and protein kinase C (PKC). These kinases phosphorylate sites in the third intracellular loop and in the C terminus of the receptor, which causes direct impairment of receptor–G1 coupling without the participation of arrestins (21–24). In the present study, we identify a cross-talk between PKC and βARK by showing that PKC directly phosphorylates βARK and causes its activation by translocation to the membrane. These studies were initiated by attempts to investigate whether βARK can also phosphorylate receptors that couple via G1 to phospholipase C, such as the αβγ-adrenergic receptor (αβγ-AR), and whether, therefore, activation of these receptors would also induce translocation of βARK to the membrane as had been shown for G1-coupled receptors. While we found no evidence for such a type of translocation, we discovered that αβγ-AR regulates βARK via activation of PKC.

MATERIALS AND METHODS

Generation of CHO Cells Stably Expressing the αβγ-AR. An expression plasmid for the hamster αβγ-AR, pBC-α1 (25), was transfected with pSV2-Neo into CHO-10001 by using the transfection reagent N-[1(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethyl sulfate (DOTAP, Boehringer Mannheim), and positive clones were selected by growing the cells in the presence of Geneticin (800 μg/ml) (GIBCO). Expression levels of αβγ-ARs were quantitated by radioligand binding with [3H]prazosin (New England Nuclear). A clone stably expressing the receptors at 0.39 ± 0.04 pmol/mg of membrane protein was used in all experiments. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) fetal calf serum.

Abbreviations: βARK, β-adrenergic receptor kinase; αβγ-AR, αβγ-adrenergic receptor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-aceitate; G protein, guanine nucleotide binding protein; GST, glutathione S-transferase.

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Transplantation of 293 Cells with βARK-1 and α1B-AR. An expression plasmid for the human βARK-1 was constructed by cloning the coding sequence of human βARK-1 (26) behind the cytomegalovirus promoter in the vector pBC-CMV-SK (27). This vector was transfected alone or with pBC-α1 into human embryonic kidney 293 cells as described by Chen and Okayama (28). Transfection efficiencies were checked with plasmids coding for β-galactosidase and were 90-100%. The cells were used for stimulation experiments 2 days after transfection. At this time, βARK activity was ~22 ± 1.5 times higher than in nontransfected cells, and α1B-ARs were expressed at ~2 pmol/ml of membrane protein.

Stimulation of Cells with Epinephrine and Phorbol Ester. The cells were grown in 3-cm Petri dishes to a confluency of ~70%. The fetal calf serum-containing medium was washed away and replaced by 3 ml of serum-free DMEM. (-)-Epinephrine, phorbol 12-myristate 13-acetate (PMA), and other compounds were added to the indicated concentrations and left on the cells for 0.5–30 min. The stimulation was stopped by removal of the medium and addition of 3 ml of ice-cold phosphate-buffered saline, which was also used for two similar washing steps. Subsequently, the cells were scraped off in 20 mM Tris-HCl, pH 7.4/50 mM NaCl/2 mM EDTA and disrupted with an Ultraturrax device. Intact cells and cell debris were removed by centrifugation at 500 × g for 5 min, and the supernatant was separated into a cytosolic and a particulate membrane fraction by centrifugation at 130,000 × g for 30 min. In some experiments, the supernatant fraction was adsorbed on DEAE-Sepharose to reduce the level of other kinases and the remaining supernatant containing βARK was then concentrated on Amicon-30 devices.

Determination of βARK Activity. βARK activity was determined by using urea-treated rod outer segments (50 pmol of rhodopsin) as the substrate as described (29) with or without 50 nM purified G-protein βγ-subunits. The incubation was carried out at 30°C for 15 min. The 32P incorporation into rhodopsin was determined by excision of the rhodopsin band after SDS/polyacrylamide gel electrophoresis and Cerenkov counting. Alternatively, βARK activity was determined with the peptide RRREEEEESAAA (1.5 mM) as the substrate according to Onorato et al. (30).

Determination of βARK Immunoreactivity. An antiserum against βARK-1 was generated by immunization of rabbits with purified recombinant βARK-1. βARK immunoreactivity was measured by Western blot analysis using this antiserum (1:2000 dilution) and peroxidase-coupled secondary antibodies (Dianova) plus ECL reagents (Amersham).

Phosphorylation of Purified βARK-1 by Purified PKC. A PKC preparation purified from rat brain was prepared according to Kikkawa et al. (31). Approximately 250 ng of this preparation (~3 pmol of PKC) was incubated with 10 pmol of purified recombinant βARK-1 in the presence of urea-treated rod outer segments (2 μg of protein) or 4 μg of crude phosphatidylcholine (type II-S from soybean) vesicles (32) in 20 mM Tris-HCl, pH 7.4/2 mM EDTA/8 mM MgCl2/50 μM CaCl2, containing 1 μM PMA plus 100 μM [γ-32P]ATP (~500 cpm/pmol). The incubation was done at 30°C for 45 min. Proteins were resolved by SDS/polyacrylamide gel electrophoresis and visualized by autoradiography. 32P incorporation was quantitated by Cerenkov counting of the excised bands.

Expression and Purification of the C Terminus of βARK-1. The cDNA sequence coding for the C terminus of human βARK-1 (amino acids 552–689) was cloned into the vector pGEX1AT (Pharmacia), which allowed its expression as a glutathione S-transferase (GST) fusion protein and its purification over glutathione-agarose using 10 mM glutathione for the elution. The purity of these preparations was >95%.

Purification of Proteins. G-protein βγ subunits were purified from bovine brain according to Sternweis and Robishaw (33). Human βARK-1 was expressed in SF9 cells and purified according to Söhlemann et al. (29). The concentration of these proteins was determined according to Bradford (34).

RESULTS

To investigate whether stimulation of α1B-AR altered the activity or cellular distribution of βARK, we generated a CHO-cell line stably expressing the α1B-AR at a density of 0.39 ± 0.04 pmol/mg of membrane protein. Functional activity of these receptors was demonstrated by the ability of epinephrine to enhance inositol phosphate production >5-fold, whereas no such effect was observed in nontransfected CHO cells (data not shown). In these cells, we examined the effects of stimulation of the α1B-AR with epinephrine for various times on the cytosolic βARK activity. In contrast to the reduction of cytosolic βARK activity caused by stimulation of β2-adrenergic receptors (11), stimulation of α1B-AR in these cells resulted in a rapid and sustained increase of cytosolic βARK activity (Fig. 1). This increase occurred as early as 30 sec after the addition of epinephrine, was maximal after 1 min with an increase by ~130%, and was maintained for about 20 min. Mediation of this effect by the transfected α1B-AR was suggested by the observations that it was absent in nontransfected CHO cells, that it occurred with an appropriate EC50 value of ~10 μM, and that it could be blocked by the α1B-AR antagonist prazosin (data not shown).

To be able to monitor the subcellular distribution of βARK immunoreactivity, we generated 293 cells transiently expressing both α1B-AR and βARK-1. In these cell lines, stimulation of α1B-AR resulted in a similar increase of cytosolic βARK activity (data not shown). Direct activation of PKC, the target kinase of the α1B-AR, with 1 μM PMA for 20 min caused an increase of specific βARK activity in the cytosol, but at the same time a marked reduction of the cytosolic βARK immunoreactivity (Fig. 2). The reduction of βARK immunoreactivity was >50% after 20 min (Fig. 2A); a concomitant increase

![Fig. 1](image-url)
of membrane-associated βARK could not be reliably detected, since in these cells basal βARK immunoreactivity in the membrane fraction was too high. The activity of the remaining cytosolic βARK was increased 2.3-fold (Fig. 2B).

These data suggested that activation of PKC might lead to activation of βARK and concomitantly to a reduction of cytosolic βARK. We then investigated whether such effects might be due to a direct interaction between the two kinases. Coincubation of PKC purified from rat brain with purified recombinant βARK-1 in the presence of phospholipid vesicles and phorbol ester led to an enhanced phosphorylation of an ~80-kD band, which contains both PKC and βARK-1 (Fig. 3A). The phosphorylation in the presence of both kinases was much larger than the sum of phosphorylation of each kinase alone. Precipitation of βARK-1 with a specific antiserum after the reaction indicated that the phosphorylation did indeed occur on βARK-1 (Fig. 3B). The stoichiometry of phosphorylation of βARK-1 in these experiments was calculated at 0.86 ± 0.27 phosphates per βARK-1. PKC-catalyzed phosphorylation of βARK-1 was confirmed by the observation that the PKC inhibitor staurosporine markedly reduced this phosphorylation (Fig. 3C).

The PKC-catalyzed phosphorylation of βARK-1 resulted in its activation when rhodopsin was used as its substrate: The phosphorylation of rhodopsin by βARK-1 was markedly increased in the presence of PKC (Fig. 4). This increase was independent of the enhancing effects of G-protein βy subunits in this assay, since PKC caused an increase of 2.9 ± 0.6 times in the absence, and an increase of 2.4 ± 0.4 times in the presence of βy subunits. Again, the enhancement was inhibited by staurosporine indicating that it was caused by PKC-catalyzed phosphorylation. Heparin, a nonselective βARK inhibitor, reduced the phosphorylation of rhodopsin to the low levels seen with PKC alone. This suggests that the enhancing effects of PKC were mediated via βARK-1 and that direct phosphorylation of rhodopsin by PKC was modest.

To test whether these effects were due to an enhancement of βARK-1 catalytic activity, the soluble peptide RRREEEEEEAAA was used as the substrate. In these assays, PKC caused no activation of βARK-1 (Table 1). In fact, the presence of PKC even caused a reduction in the βARK-1-mediated phosphorylation of the peptide. This suggests that the PKC-mediated activation seen with rhodopsin as the substrate was not due to enhanced catalytic activity but was possibly due to improved membrane targeting.

The C terminus of βARK-1 is the domain responsible for membrane attachment since it binds both G-protein βy subunits and phosphatidylinositol bisphosphate (19). If PKC affects the membrane attachment, then it would appear likely that phosphorylation of βARK-1 occurs at the C terminus. A clear consensus sequence for PKC remains to be defined; according to the criteria of Kennelly and Krebs (35), the C terminus of βARK-1 contains four suboptimal PKC phosphorylation motifs with arginine or lysine residues 1–3 positions N- or C-terminal of a serine/threonine residue. Indeed, the βARK-1 C terminus expressed as a GST fusion protein proved to be a very good substrate for PKC (Fig. 5). The stoichiometry of phosphorylation was similar to that of the entire βARK-1, suggesting that a single relevant phosphorylation site for PKC is located in this C terminus.

**DISCUSSION**

Activation of different types of G<sub>i</sub>-coupled receptors causes a translocation of βARK activity from the cytosol to membranes (11, 36, 37). Since βARK has also been shown to phosphorylate receptors coupled to other G proteins, such as the G<sub>i</sub>-coupled β2-adrenergic and the m2-muscarinic acetylcholine receptors and the G<sub>i</sub>-coupled substance P receptors (38–40), we speculated that stimulation of the G<sub>i</sub>-coupled α<sub>1B</sub>-AR might cause a similar translocation of βARK. Such a translo-

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**FIG. 2.** Effects of PMA on the cytosolic βARK content and activity in 293 cells transiently expressing βARK-1. 293 cells were transiently transfected with a βARK-1 expression vector and 2 days later exposed to 1 μM PMA for 20 min. Cytosolic fractions were prepared as described for Fig. 1 and assayed for βARK immunoreactivity in Western blots (4). Cytosolic fractions containing equal amounts of βARK (as estimated from similar Western blots) were then assayed for βARK activity by using rhodopsin as the substrate as described for Fig. 1 and expressed as activity per immunoreactivity determined by laser densitometry in arbitrary units (B). Data are the mean ± SEM (n = 6).

**FIG. 3.** Phosphorylation of purified βARK-1 by purified PKC. Purified recombinant βARK-1 (10 pmol) was phosphorylated by 250 ng of a PKC preparation purified from rat brain in the presence of 4 μg of phospholipid vesicles and 1 μM PMA. The proteins were resolved by SDS/polyacrylamide gel electrophoresis and visualized by autoradiography, and 32P incorporation was determined by Cerenkov counting of the excised bands. (A) Protein band of ~80 kDa, which contains both βARK-1 and PKC. (B) Same band after immunoprecipitation with a βARK-1 antiserum. (C) 32P incorporated into βARK-1 without or with 100 nM staurosporine. Data are the mean ± SEM (n = 3).

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Fig. 4. Activation of purified βARK-1 by purified PKC. Purified recombinant βARK-1 (10 pmol = 5 nM) was incubated with 250 ng of a PKC preparation purified from rat brain in the presence of urea-treated rod outer segments (50 pmol of rhodopsin), 1 μM PMA, and [γ-32P]ATP. The proteins were resolved by SDS/polyacrylamide gel electrophoresis, the rhodopsin band was visualized by autoradiography, and 32P incorporation was determined by Cerenkov counting of the excised band. (A) Rhodopsin band after phosphorylation with βARK-1 and/or PKC in the absence of inhibitors or with 100 nM staurosporine or 100 nM heparin. (B) Rhodopsin phosphorylation obtained with βARK-1 with and without PKC in the absence or presence of 50 nM G-protein βγ subunits. Data are the mean ± SEM (n = 4).

Fig. 5. Phosphorylation of the C-terminal domain of βARK-1 by purified PKC. The C terminus of βARK-1 (amino acids 552–689) was expressed in Escherichia coli as a GST fusion protein and purified. GST alone produced similarly served as a control. Each protein (13 pmol) was incubated with 250 ng of a PKC preparation purified from rat brain in the presence of 4 μg of phospholipid vesicles and 1 μM PMA. The proteins were then resolved by SDS/polyacrylamide gel electrophoresis, and the 32P incorporation was visualized by autoradiography. Arrows indicate the positions of PKC, the GST–βARK-1–C terminus (CT) fusion protein, and GST.

with inhibitors and phosphorylation of the isolated C terminus of βARK-1 indicated that the PKC-induced increase in phosphorylation and activity of βARK-1 was indeed due to PKC-catalyzed phosphorylation and not due to PKC-induced enhancement of βARK-1 autophosphorylation.

Most of the C terminus of βARK is formed by a pleckstrin homology domain, which together with a C-terminally adjacent α-helix has been implicated in binding to G-protein βγ subunits as well as to phosphatidylinositol bisphosphate (19). Two other kinases that contain a pleckstrin homology domain, Bruton tyrosine kinase and RAC (related to A- and C-kinase) kinase, have been shown to interact with PKC (41, 42). In the case of Bruton tyrosine kinase, this interaction has been studied also at the functional level and has been found to cause decreased enzymatic activity of the kinase. Thus, the activation of βARK by PKC must be caused by another mechanism. Indeed, the catalytic activity of βARK was even decreased when a soluble peptide was used as the substrate. This suggests that the increased activity toward rhodopsin, a membrane-bound substrate, might be due to enhanced membrane targeting of the kinase. This hypothesis is compatible with the observation that in intact cells βARK levels in the cytosol were indeed reduced after activation of PKC.

Our findings suggest that in addition to substrate availability and membrane attachment, there is a third level of regulation for βARK: phosphorylation-mediated membrane translocation. Such a chain of phosphorylation (PKC → βARK → receptor) can explain the observation that desensitization of odorant receptors can be inhibited either by heparin or by PKC inhibitors (43). Furthermore, PKC-mediated regulation of βARK connects the desensitization feedback loop mediated by βARK with the effector pathway of PKC. This adds another level of complexity to the many mechanisms that operate in G-protein-mediated transmembrane signaling.

Table 1. Effect of PKC on peptide phosphorylation by βARK-1

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<tr>
<th>Phosphate incorporation rate, fmol of P_i per min per pmol of βARK-1</th>
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<tr>
<td>βARK-1 alone</td>
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<td>βARK-1 + PKC</td>
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Peptide RRREEEESAA (1.5 mM) was incubated with βARK-1 (10 pmol) in the presence of 100 μM ATP (~270 cpm/pmol) and with or without 250 ng of purified rat brain PKC at 30°C for 30 min. The phosphorylation of the peptide was determined. The phosphate incorporation rate is expressed as the mean ± SEM (n = 6).

Note Added in Proof. Chuang et al. (J. Biol. Chem. 270, 18660–18665) have reported phosphorylation of βARK by PKC and a resultant increase of rhodopsin phosphorylation, which they attribute to direct activation of βARK.

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