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# PKCβII inhibits the ubiquitination of β-arrestin2 in an autophosphorylation-dependent manner

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ABSTRACT

**GPCR kinase 2 (GRK2)/β-arrestins and protein kinase A (PKA)/protein kinase C (PKC) mediate homologous and heterologous regulations of GPCRs, respectively. Conventional protein kinase C enzymes (PKCs), as exemplified by PKCβII, selectively inhibit internalization of dopamine $D_2$ receptor and $\beta_2$ adrenoceptor in a β-arrestin- but not GRK2-dependent manner. PKCβII interacts with β-arrestin2 upon autophosphorylation at T250, and inhibits the receptor internalization by decreasing the ubiquitination of β-arrestin2. PKCβII interferes with the interaction between β-arrestin2 and MDM2 in the cytosol, resulting in the redistribution of MDM2 to the nucleus. Subsequently, deubiquitination of β-arrestin2 and inhibition of agonist-induced receptor internalization follow. Thus, our study suggests that the extent of β-arrestin ubiquitination and the autophosphorylation status of PKCs determine PKCβII-mediated inhibition of homologous regulatory processes of GPCRs.**


## 1. Introduction

The desensitization of G protein-coupled receptors (GPCRs) was originally defined as a diminishment in receptor responsiveness through receptor phosphorylation by second messenger-dependent protein kinases, such as protein kinases A and C (PKA and PKC) [1–3]. PKA and PKC can phosphorylate both agonist-occupied and unoccupied GPCRs without discrimination (heterologous). Later, a universal mechanism for desensitizing GPCRs was proposed (homologous, agonist-induced). Homologous desensitization is known to be mediated by the coordinated actions of two families of proteins, the G protein-coupled receptor serine/threonine kinases (GRKs) and the arrestins [4–6]. It has been suggested that an agonist-occupied receptor has a specific conformation that allows for the binding of the GPCR kinase 2 (GRK2) [7].

It is generally accepted that homologous and heterologous desensitization pathways operate independently. However, there has been some evidence to indicate functional interactions between the two pathways. For example, the enzymatic activities of GRK2 were shown to be increased through phosphorylation on the 29th serine residue by PKCα, γ, and δ in vitro [8]. Subsequent studies suggested that PKC activates GRK2 by enhancing its translocation to the plasma membrane [9,10]. On the other hand, some studies have shown that PKCs phosphorylate Raf kinase inhibitor protein (RKIP), which in turn inhibits GRK2 activities [11,12].

Involvement of β-arrestins in the PKC-mediated receptor phosphorylation was suggested in $D_2$ dopamine receptor [13], the extracellular $Ca^{2+}$-sensing receptor [14], and the δ-opioid receptor [15]. In addition, it was shown that β-arrestins inhibit signaling through the Gq-coupled receptor by degrading diacylglycerol to phosphatidic acid [16]. Thus, there seem to be complicate functional interactions among PKC, GRK2, and β-arrestins.

In our preliminary studies to understand the functional interactions between homologous and heterologous regulation, we noticed that conventional PKCs inhibited the agonist-induced internalization of $\beta_2$ adrenoceptor and $D_2$ receptor. These results are opposite to the previously reported roles of PKCs on the homologous regulatory processes of GPCRs [8,10]. We conducted more systemic subsequent studies to outline a novel pathway that is

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involved in the PKC-mediated inhibition of homologous regulatory pathways of GPCRs.

## 2. Materials and methods

### 2.1. Materials

DA, ISO, PMA, 4α-PMA, anti-FLAG-conjugated agarose beads, antibodies against actin and green fluorescent protein (GFP), horseradish peroxidase (HRP)-labeled secondary antibodies, and glutathione beads were purchased from Sigma/Aldrich Chemical (St. Louis, MO, USA). [$^3$H]-Sulpiride (87 Ci/mmol) and [$^3$H]-CGP-12177 (41.7 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA, USA). Antibodies against the hemagglutinin (HA) epitope, MDM2, and GRK2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Gö6976 and Gö6983 were from Merck Millipore (Billerica, MA, USA). Antibodies against PKCβ and phosphorylated PKCβ were purchased from Abcam (Cambridge, MA, USA). Antibodies against β-arrestins were provided by Dr. Robert Lefkowitz (Duke University, Durham, NC, USA).

### 2.2. Cell culture and transfection

Human embryonic kidney 293 (HEK-293) cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C/5% $CO_2$. Transfections were performed using the calcium phosphate precipitation method or with polyethylenimine (Polyscience, Warrington, PA, USA).

### 2.3. Plasmid constructs

Plasmids containing inserts corresponding to wild-type human $D_2R$ and $\beta_2AR$, and rat β-arrestin1 and β-arrestin2 were generated previously [17–19]. These receptors were conjugated to the Flag epitope at the N-terminus. Using polymerase chain reactions (PCRs), we prepared GFP-, Flag-, and HA-tagged PKCβII, Flag-tagged MDM2, and GFP-tagged MDM2. T250A-PKCβII, Flag-tagged T250A-PKCβII, K11/12R-β-arrestin2, and Flag-tagged K11/12R-β-arrestin2 were prepared by site-directed mutagenesis. The generation of PKCα, PKCβI, PKCβII, PKCε, and PKCζ conjugated to GFP, along with PKCα, PKCβII, PKC γ, and PKC δ fused to HA has been described previously [20,21]. The shRNAs for GRK2, MDM2, and PKCβII were described previously [22–24]. β-Arrestin1 and β-arrestin2 shRNAs were provided by Dr. Lan Ma (Fudan University, Shanghai, China). We used PCR methods to prepare GST-conjugated C1, C2, and KD domains of PKCβII, and GFP-tagged C1 and C2 domains of PKCβII. We also produced GST-conjugated full-length β-arrestin2, along with N, C, and CCT (C domain plus carboxyl) domains by polymerase chain reaction.

### 2.4. Endocytosis assays

Endocytosis of $D_2R$ and $\beta_2AR$ was measured based on the hydrophilic properties of [$^3$H]-sulpiride and [$^3$H]-CGP-12177 [17,25]. HEK-293 cells expressing $D_2R$ or $\beta_2AR$ were seeded at a density of $1.5 \times 10^5$ cells/well in 24-well plates 1 day after transfection. The following day, cells were rinsed and pre-incubated with 0.5 mL of pre-warmed serum-free medium containing 10 mM HEPES (pH 7.4) at 37 °C for 15 min. Cells were stimulated with 10 μM DA or ISO for 0–60 min, and then incubated with 250 μL of [$^3$H]-sulpiride (final concentration, 2.2 nM) or [$^3$H]-CGP-12177 (final concentration, 10 nM) at 4 °C for 150 min, in the absence or presence of unlabeled competitive inhibitor (10 μM haloperidol or propranolol). Cells were washed three times with the same medium and 1% SDS was added. Samples were mixed with 2 mL of Lefkofluor scintillation fluid and radioactivity was determined with a liquid scintillation analyzer.

### 2.5. Immunoprecipitation

At 48 h post-transfection, cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) for 1 h at 4 °C with gentle mixing. Supernatants were mixed with 25 μL of anti-Flag agarose beads (50% slurry) for 2–3 h. Beads were washed (3 × 10 min) with washing buffer (50 mM Tris pH 7.4, 137 mM NaCl, 10% glycerol, 1% NP-40). Immunoprecipitates were analyzed by SDS polyacrylamide gel electrophoresis (PAGE) and immunoblotted with antibodies against target proteins, with experiments repeated three or four times.

### 2.6. In vitro binding studies

The subdomains of proteins of interest were expressed in bacteria with a GST tag, as described previously [26]. Lysates of HEK-293 cells expressing the target protein were incubated with glutathione agarose beads, which bound to the GST fusion proteins. Glutathione agarose beads were washed, and the retained proteins were eluted with SDS sample buffer. Eluents were analyzed by SDS–PAGE and immunoblotted with antibodies against target proteins.

### 2.7. Immunocytochemistry

HEK-293 cells were plated on glass coverslips and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 in PBS at room temperature, and then incubated with PBS containing 3% FBS and 1% bovine serum albumin for 1 h. Cells were incubated with antibodies against FLAG and HA (1:1000 dilutions) for 1 h at room temperature. After three washes, cells were incubated with Alexa 555-conjugated anti-mouse or Alexa 647-conjugated anti-rabbit antibodies (1:500; Invitrogen, Carlsbad, CA, USA). Following three washes with washing buffer, cells were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and viewed with a laser-scanning confocal microscope (TCS SP5/ABOS/Tandem, Germany).

### 2.8. Detection of ubiquitinated β-arrestin2

FLAG-β-arrestin2 and HA-ubiquitin were transiently transfected into HEK-293 cells along with $\beta_2AR$. Cells were serum-starved for 4–6 h and then treated with 10 μM ISO for 2 min. Cell lysates were solubilized in a lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1 mM EDTA, 1% TritonX-100, 10% (v/v) glycerol, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 2 mM phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, 5 μg/mL aprotinin, 10 mM N-ethylmaleimide) and immunoprecipitated with FLAG beads. Immunoprecipitates were analyzed by SDS–PAGE and blotted with antibodies against HA and FLAG.

### 2.9. Data analysis

Values are expressed as the mean ± standard error (S.E.), with Student's *t*-test used to compare results. Comparisons among experimental groups were performed using analysis of variance (ANOVA) and Tukey's range test. A *P*-value less than 0.05 was considered statistically significant.