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Inhibitory effects of two G protein-coupled receptor kinases on the cell surface expression and signaling of the human adrenomedullin receptor

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ABSTRACT

Receptor activity-modifying protein 2 (RAMP2) enables the calcitonin receptor-like receptor (CLR, a family B GPCR) to form the type 1 adrenomedullin receptor (AM_1 receptor). Here, we investigated the effects of the five non-visual GPCR kinases (GRKs 2 through 6) on the cell surface expression of the human (h)AM_1 receptor by cotransfecting each of these GRKs into HEK-293 cells that stably expressed hRAMP2. Flow cytometric analysis revealed that when coexpressed with GRK4 or GRK5, the cell surface expression of the AM_1 receptor was markedly decreased prior to stimulation with AM, thereby attenuating both the specific [^{125}I]AM binding and AM-induced cAMP production. These inhibitory effects of both GRKs were abolished by the replacement of the cytoplasmic C-terminal tail (C-tail) of CLR with that of the calcitonin receptor (a family B GPCR) or β_2-adrenergic receptor (a family A GPCR). Among the sequentially truncated CLR C-tail mutants, those lacking the five residues 449–453 (Ser-Phe-Ser-Asn-Ser) abolished the inhibition of the cell surface expression of CLR via the overexpression of GRK4 or GRK5. Thus, we provided new insight into the function of GRKs in agonist-unstimulated GPCR trafficking using a recombinant AM_1 receptor and further determined the region of the CLR C-tail responsible for this GRK function.

1. Introduction

G protein-coupled receptors (GPCRs) are the largest family of integral transmembrane proteins and remain the largest class of therapeutic targets in medicine, accounting for one-third of all marketed pharmaceuticals [1]. GPCR kinases (GRKs) are classified into three subgroups (i.e., GRKs 1 and 7, GRKs 2 and 3 and GRKs 4, 5 and 6); GRKs 2 through 6 are also known as “non-visual GRKs” [2]. Among non-visual GRKs, GRKs 2, 3, 5 and 6 are ubiquitously expressed, although to varying degrees depending on the tissue, whereas GRK4 is predominantly found in the testes and renal tissue. These GRKs induce the desensitization and internalization of multiple GPCRs [3–5]. In many cases, intracellular signaling proteins called β-arrestins promote the intracellular trafficking of GPCRs. Specifically, GRKs 2 through 5 worsen clinical conditions, such as hypertension (GRKs 2 through 5), heart failure (GRKs 2 and 5), cardiac/vascular hypertrophy (GRK2), cardiomyopathy (GRK5) and atherosclerosis (GRK2) [6–8]. Therefore, these GRKs have attracted considerable attention for drug targeting. Recent studies have revealed that GRKs also interact with many intracellular proteins [9].

The calcitonin receptor-like receptor (CLR) is a family B GPCR that is transported from the endoplasmic reticulum to the cell surface with the help of three receptor activity-modifying proteins

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(RAMPs 1 through 3) that possess a single membrane-spanning domain [10,11]. RAMP1 forms a calcitonin gene-related peptide (CGRP) receptor together with CLR. RAMP2 or RAMP3 enables CLR to function as two adrenomedullin (AM) receptors, i.e., the AM_1 and AM_2 receptors, respectively. Adrenomedullin (AM) is a potent vasodilator that has been demonstrated to exert powerful anti-oxidative and antiatherosclerotic effects [12,13]. Like non-visual GRKs, the AM_1 receptors exhibit a wide tissue distribution; the receptors are highly expressed in the cardiovascular system [14,15]. Unlike AM_2 receptors, AM_1 receptors are crucial for fetal cardiovascular development and protect central and vascular integrity and homeostasis [16–19]; however both receptors evoke cAMP production and Ca^{2+} mobilization in response to AM stimulation [10,20].

The cytoplasmic C-terminal tail (C-tail) of human (h)CLR consists of 73 amino acid residues and contains 12 Ser/Thr residues that are potential phosphorylation sites [21], whereas the three hRAMPs consist of only 9 amino acid residues and possess two or three Ser/Thr residues [14]. However, CGRP failed to phosphorylate RAMP1 but fully induced the phosphorylation of CLR [22]. We recently demonstrated that the hCLR C-tail is crucial for AM-mediated cAMP production, interactions with both Gs and Gi, and the internalization of the AM_1 receptor but not for AM binding [23]. We also demonstrated that AM-induced receptor internalization is dependent on GRKs 2, 3 and 4 and is not dependent on Gs coupling [23]. In general, many agonists undergo the internalization of their GPCRs in a dose-dependent manner, which is clinically required for both desensitization and resensitization of the receptor signaling. During AM-induced AM_1 receptor internalization, these three GRKs have been suggested to interact with the Ser/Thr-rich region extending from Ser^{449} to Gly^{469} [23]. Although the AM and AM_1 receptor mRNAs are known to be upregulated in the cardiovascular system under conditions of various cardiovascular diseases, the receptor system cannot compensate for their conditions. To our knowledge, little is known about the relationship between the overexpression of non-visual GRKs and the cell surface expression of GPCRs prior to stimulation by their hypotensive agents. To address this issue, we investigated these effects using various mutants of the C-tail of hCLR, a family B GPCR, in HEK-293 cells that stably expressed hRAMP2, which enables CLR to function as the AM_1 receptor.

2. Materials and methods

2.1. Reagents and antibodies

The [^{125}I]hAM (specific activity 2 μCi/pmol) was produced in our laboratory [24]. The human AM was kindly donated by Shionogi & Co. (Osaka, Japan). The FITC-conjugated mouse anti-V5 monoclonal antibody (anti-V5-FITC antibody) was from Invitrogen. All other reagents were of analytical grade and obtained from various commercial suppliers.

2.2. Expression constructs

Double V5-tagged hCLR (V5-hCLR) [25] was cloned into the expression vector pCAGGS/Neo [20] to yield pCAGGS-V5-hCLR. Progressive truncation of the V5-hCLR C-tail was accomplished using 3'-primers that introduced translational stop codons at the desired positions [23]. The insert-negative isoform of the human calcitonin receptor (CTR) was cloned in our laboratory [26]. The human β_2-adrenergic receptor (β_2-AR, GenBank accession no. NM_000024) was cloned from cDNA obtained from a human cerebellum (Clontech) using PCR with appropriate primers. Two chimeras, i.e., V5-CLR/CTR and V5-CLR/β_2-AR, were generated by replacing the CLR C-tail with the corresponding sequence from CTR or β_2-AR using a restriction site (*Avi* II site) [27] that was situated in the middle of a putative eighth helix (helix 8) [27]. The restriction site was newly introduced without altering the amino acid sequence of the receptor because the C-tails of CLR, CTR and β_2-AR contain none of the common sites. The aforementioned PCR products were all sequenced using an Applied Biosystems 310 Genetic Analyzer (Foster City, CA, USA).

All of the non-visual GRKs (GRKs 2 through 6) were obtained from the lab of Stephan S.G. Ferguson [28]. Using SDS-PAGE, the protein expression levels of the total cellular GRKs 2 through 6 that transiently transfected into HEK-293 cells were GRK5 > GRK6 > GRK2, GRK3, GRK4 [28].

2.3. Cell culture and DNA transfection

Human embryonic kidney (HEK)-293 cells stably expressing hRAMP2 [23] were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 100 μg/ml hygromycin B (Wako, Osaka, Japan) at 37 °C under a humidified atmosphere of 95% air/5% CO_2.

The transient transfection of HEK-293 cells stably expressing hRAMP2 was accomplished using Lipofectamine™ and Plus™ reagents (Invitrogen). Briefly, the cells were seeded into 12-well (for flow cytometric analysis) or 24-well plates (for binding and cAMP assays); upon reaching 70–80% confluence, the cells were transfected with empty vector (pCAGGS/Neo) (*Mock*) or wild-type (WT) or mutant V5-tagged constructs. DNA complexing was accomplished by incubating the cells for 4 h in OptiMEM 1 medium containing the plasmid DNAs and Plus and Lipofectamine reagents (see Ref. [27]). The ratio of transfection amounts of V5-CLR to each of the GRKs was 10:4 and/or 10:1; in both cases, total transfection amounts were equal because empty vector DNAs for all GRKs tested were added in accordance with reduced transfection amounts of GRK. All of the following experiments were performed 36–48 h after transfection.

2.4. Flow cytometric analysis

Flow cytometry was used to assess the cell surface expression and whole cell expression of the V5-tagged receptor proteins. To evaluate of cell surface expression of the receptors, following the cotransfection of the indicated V5-tagged cDNAs into the hRAMP2-expressing HEK-293 cells in 12-well plates, the cells were replaced with DMEM containing 0.5% FBS 24 h prior to the flow cytometric analysis to eliminate the effect of FBS. Thereafter, the cells were washed once with PBS and then non-enzymatically harvested with ice-cold FACS [20]. After centrifugation, the cells were resuspended in FACS buffer and labeled with anti-V5-FITC antibody (diluted to 1:1000 in FACS buffer) for 2 h at 4 °C in the dark. To evaluate the whole cell expression of the receptors, the cells were first permeabilized using IntraPrep™ reagents (Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions [29] and then incubated with anti-V5-FITC antibody (diluted to 1:1000 in FACS buffer) for 15 min at room temperature in the dark. Following two successive washes, the cells were subjected to flow cytometry in an EPICS XL flow cytometer (Beckman Coulter). The cell surface expression frequency of each V5-tagged receptor (% of cells) was analyzed using EXPO 2 software (Beckman Coulter) [20]. FITC fluorescence was elicited at 488 nm, and emission was monitored at 530 nm. In this text, "cell surface expression of the receptors" means "the percentage of FITC-positive cells (V5-CLR-expressed cells)" in the 10,000 cells collected during flow cytometry.