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Regulation of G protein signaling by the 70 kDa heat shock protein

William K. Lim [a,*], Kimon C. Kanelakis [b,1], Richard R. Neubig [b,c]

[a] Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak, 93150 Kuching, Sarawak, Malaysia
[b] Department of Pharmacology, The University of Michigan, Ann Arbor, MI 48109-5632, USA
[c] Department of Internal Medicine/Cardiovascular Medicine, The University of Michigan, Ann Arbor, MI 48109-5632, USA

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ABSTRACT

G protein-coupled receptors (GPCRs) transduce extracellular signals to the interior of the cell by activating membrane-bound guanine nucleotide-binding regulatory proteins (G proteins). An increasing number of proteins have been reported to bind to and regulate GPCRs. We report a novel regulation of the alpha$_{2A}$ adrenergic receptor (α_{2A}-R) by the ubiquitous stress-inducible 70 kDa heat shock protein, hsp70. Hsp70, but not hsp90, attenuated G protein-dependent high affinity agonist binding to the α_{2A}-R in Sf9 membranes. Antagonist binding was unchanged, suggesting that hsp70 uncouples G proteins from the receptor. As hsp70 did not bind G proteins but complexed with the α_{2A}-R in intact cells, a direct interaction with the receptor seems likely. In the presence of hsp70, α_{2A}-R-catalyzed [^{35}S]GTPγS binding was reduced by approximately 70%. In contrast, approximately 50-fold higher concentrations of hsp70 were required to reduce agonist binding to the stress-inducible 5-hydroxytryptamine$_{1A}$ receptor (5-HT$_{1A}$-R). In heat-stressed CHO cells, the α_{2A}-R was significantly uncoupled from G proteins, coincident with an increased localization of hsp70 at the membrane. The contrasting effect of hsp70 on the α_{2A}-R compared to the 5-HT$_{1A}$-R suggests that during stress, upregulation of hsp70 may attenuate signaling from specific GPCRs as part of the stress response to foster survival.

1. Introduction

The seven transmembrane domain G protein-coupled receptor (GPCR) family constitutes the largest class of cell surface receptors in mammals [1]. The intracellular loops of these receptors bind G proteins, which consist of an α subunit and a βγ heterodimer [2]. In the classical model of GPCR signaling, the binding of an extracellular ligand to receptors catalyzes the exchange of GDP for GTP on the α subunit, reducing the affinity of α for both βγ and receptor [3]. Activated G proteins can modulate the activities of effector molecules such as adenylyl cyclase, phospholipases or ion channels [4]. Upon GTP hydrolysis, the α-βγ heterotrimer is re-formed for receptor coupling. The G protein signaling system is regulated by a host of diverse proteins that interact with components at the plasma membrane [5], receptor or G protein, as was recently reviewed [6,7].

GPCRs are conserved across species, from the unicellular yeast [8] and plants [9] to higher organisms. Likewise, heat shock proteins (HSPs) are found in bacterial, plant and animal species [10]. There are five human HSP families named after their molecular mass, of which HSP70 (hsp70, HSPA) is the best studied, followed by HSP90 (hsp90, HSPC) [11]. The two most prominent members of the hsp70 family are the constitutively expressed 73 kDa heat shock cognate (hsc73, hsc70, HSPA8) and stress-inducible 72 kDa heat shock protein (hsp72, HSP1A). Both families exhibit similar biochemical activities. They are molecular chaperones that assist in protein folding, stabilization, translocation and degradation [12]. An increasing number of eukaryotic proteins are known to be regulated through transient association with hsp70, including steroid hormone receptors [13], kinases [14] and transcription factors [15]. Client proteins bind to and are released from hsp70 in an ATP-dependent manner with the help of cochaperones. ATP-bound hsp70 has an open conformation for substrate binding but hydrolysis to an ADP-bound state allows high affinity binding until ADP is exchanged for ATP [16]. Various

Abbreviations: GPCR, G protein-coupled receptor; G proteins, heterotrimeric guanine nucleotide-binding regulatory proteins; A$_1$-R, adenosine A$_1$ receptor; HSP, heat shock protein; HSP70/hsp70, 70 kDa family of heat shock proteins; 5-HT$_{1A}$-R, 5-hydroxytryptamine$_{1A}$ receptor; α_{2A}-R, alpha$_{2A}$ adrenergic receptor; hsc73, heat shock cognate 73 kDa; hsp72, heat shock protein 72 kDa; hsp90, heat shock protein 90 kDa; ADA, adenosine deaminase; YOH, yohimbine; PIC, *p*-iodoclonidine; 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)tetralin; CHO, Chinese hamster ovary; Sf9, *Spodoptera frugiperda*; DTT, dithiothreitol; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; GTPγS, guanosine 5′-(3-o-thiotriphosphate); hsp40, heat shock protein 40 kDa; hsp60, heat shock protein 60 kDa; AMF, aluminum magnesium fluoride; PAGE, polyacrylamide gel electrophoresis; HA, human influenza hemagglutinin; HRP, horseradish peroxidase; PVDF, polyvinylidene fluoride.

* Corresponding author at: Department of Paraclinical Sciences, Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak, Lot 77, KTLD Section 22, Jalan Tun Zaidi Adruce, 93150 Kuching, Sarawak, Malaysia. Tel.: +60 82 292 252; fax: +60 82 422 564.
E-mail address: kslim@fmhs.unimas.my (W.K. Lim).

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physiological and environmental insults induce the expression of HSPs, including elevated temperatures, classically referred to as heat shock. In mammals there is increased tissue expression of HSPs after stress events such as ischemia/reperfusion [17] and surgery [18]. Induced hsp70 is cytoprotective, mediating survival by preventing protein aggregation and facilitating protein refolding or disposal [19]. More recently, hsp70 in tumor cell lines has been reported to translocate to the plasma membrane and also secreted extracellularly as a cytokine to activate the immune system [20,21].

Although intracellular HSPs are mostly cytosolic, certain HSPs are expressed at the cell surface. Hsp70 and hsp90 were found to be immobile components at cell membranes of a human monocytic cell line [22]. The HSP70 family members mitochondrial hsp70 (mt-hsp70), hsc73 and hsp72 are expressed at the surface of CHO cells [23,24]. Heat shock has been reported to translocate a subset of hsp70 from the cytosol to the plasma membrane of erythrocytes [25]. These results suggest that the ubiquitous and stress-inducible hsp70 may play important but yet undefined roles at the plasma membrane.

There is evidence that HSPs can regulate cell surface receptors. Hsp90 and to some extent hsp70 were shown to be required for insulin receptor-mediated mitogenesis [26]. Hsp90 binds and regulate signaling from the PAR-1 protease-activated receptor [27], CB2 cannabinoid receptor [27,28] and trafficking of the α_{2C} adrenergic receptor [29]. The adenosine A_1 receptor (A_1-R) was reported to co-immunoprecipitate with hsc70 [30]. Reconstitution of hsp70/hsc70 with purified A_1 receptors led to the suppression of both agonist and antagonist binding in a manner that was reversed by adenosine deaminase (ADA), a co-factor required for G protein coupling to the A_1 receptor [30]. The unique role of ADA in A_1-R function and the dependence of hsc70 effects on ADA make it difficult to know if the effect of hsc70 is relevant to GPCR signaling in general.

In this report we show that hsp70 reduces agonist but not antagonist binding to the alpha$_{2A}$ adrenergic receptor (α_{2A}-R), a prototypical GPCR, consistent with an uncoupling of receptor from G proteins. Hsp70 did not bind G proteins in a protein–protein interaction assay but was in complex with the α_{2A}-R in whole cells, suggesting a direct interaction with α_{2A}-R. In contrast to the α_{2A}-R, hsp70 requires approximately 50-fold higher concentrations to inhibit agonist binding to the 5-HT_{1A} receptor (5-HT_{1A}-R). In intact cells, heat stress attenuated receptor-G protein coupling in parallel with translocation of hsp70 to the plasma membrane. These results demonstrate a novel regulation of GPCRs by hsp70 and suggest that in cellular stress, hsp70 acts as a switch to turn off signaling from specific GPCRs.

2. Material and methods

2.1. Material

[^{125}I] *p*-iodoclonidine (PIC, 2200 Ci/mmol), [^{3}H] yohimbine (YOH 80 Ci/mmol) and [^{3}H] 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, 125 Ci/mmol) were from Perkin Elmer (Waltham, MA). UK14,304 was from Pfizer (Sandwich, England), urea was from Fisher Scientific (Fair Lawn, NJ) while YOH, 5HT and Gpp(NH)p were from Sigma (St. Louis, MO). Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). SDS-PAGE gels were stained with Gelcode Blue Stain Reagent (Thermo Fisher Scientific, Rockford, IL). Protein molecular weights in Western blotting were estimated using SuperSignal Enhanced Molecular Weight Protein Ladder (Thermo Fisher Scientific). All other reagents were of reagent grade or better.

2.2. Membrane preparation

We have previously described the stable transfection of a porcine α_{2A}-R construct in CHO cells, and construction of baculoviruses encoding the same receptor [31]. Baculoviruses encoding the human 5-HT_{1A}-R were provided by Dr. David Manning (University of Pennsylvania). Isolation of membranes (P2 pellet) from CHO or Sf9 cells expressing receptor was performed as previously described [31]. To uncouple receptor from endogenous G proteins prior to reconstitution, membranes were washed with 7 M urea as previously reported [32]. Briefly, urea solutions were freshly prepared by dissolution into 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$ and 1 mM EDTA (TME) with protease inhibitors 2 μg/mL aprotinin, 10 μg/mL leupeptin, 0.1 mM PMSF and 20 μg/mL benzamidine. Membranes were incubated with urea on ice for 30 min then collected by centrifugation at 140,000 *g* for 30 min. A second wash with TME buffer was performed before membranes were aliquoted and snap frozen in liquid nitrogen.

2.3. Protein purification

G protein α_{i1} and $\beta_1\gamma_2$ subunits were synthesized in Sf9 cells from 2.5–4 l cultures and purified according to published protocols [33]. Briefly, (i) α_{i1}: Sf9 cells were co-infected with baculoviruses encoding his$_6$-β_1, his$_6$-γ_2 and α_{i1}. Cells were burst by nitrogen cavitation and extracted with 1% cholate. Cell extract was loaded onto a 0.8 cm × 4 cm column (Poly-Prep, Bio-Rad, Hercules, CA) filled with 6 mL of Nickel-NTA agarose resin (Qiagen, Valencia, CA). α_{i1} was isolated from βγ by washing with AMF buffer (30 μM $AlCl_3$, 50 mM $MgCl_2$, and 10 mM NaF). (ii) $\beta_1\gamma_2$: Sf9 cells were infected with baculoviruses expressing his$_6$-α_{i1}, β_1 and γ_2, and $\beta_1\gamma_2$ was purified and eluted as above. (iii) his$_6$-labeled G proteins: Sf9 cells were infected with either his$_6$-α_{i1} or β_1 and his$_6$-γ_2. For elution, the column was washed sequentially with buffer containing 20 mM imidazole, then 40 mM imidazole, and then his$_6$-tagged G proteins were eluted with two washes of 150 mM imidazole. For βγ preparations, this eluate was applied to a Mono Q HR 5/5 cation exchange column (GE Healthcare, Little Chalfont, UK) and eluted as described [33]. Finally, G proteins were concentrated using an Ultrafree-15 centrifugal filter device (Millipore, Bedford, MA) to ~2–3 mg/mL. βγ preparations were suspended in 20 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 1 mM DTT and 0.6% CHAPS while α subunits were in 20 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT. βγ protein concentration was estimated by densitometry of the β band obtained from ~1 μg of this Sf9-expressed βγ loaded onto a 10% SDS-PAGE gel and stained with a Coomassie-based dye. Bovine brain βγ (concentration determined by Amido Black staining) was used as a standard. Protein concentration of α subunits was determined by the Bradford assay and activity monitored by [^{35}S] GTPγS binding as described [34]. Hsc/hsp70 from rabbit reticulocyte lysate was purified by sequential chromatography on DE52 DEAE-cellulose, hydroxylapatite, and ATP-agarose while hsp90 was isolated using DE52 DEAE-cellulose followed by hydroxylapatite chromatography as previously described [35]. Both proteins were dialyzed into HKD buffer (10 mM Hepes, pH 7.4, 100 mM KCl and 5 mM DTT). Protein concentration was determined using the Bradford assay. Purity of protein preparations was examined by SDS-PAGE. The activity of purified YDJ-1, hsp70 and hsp90 was verified by the ability to reconstitute high affinity steroid binding to the glucocorticoid receptor as previously described [36]. Purified proteins were aliquoted, snap frozen in liquid nitrogen and stored at −80 °C.

2.4. Radioligand and [^{35}S] GTPγS binding

Binding of the α_{2A}-R expressed in CHO or Sf9 membranes with the radiolabeled ligands [^{125}I] PIC, [^{3}H]YOH, [^{3}H]8-OH DPAT and binding of α subunits by the non-hydrolyzable GTP analog, [^{35}S]GTPγS, were measured as described [32]. Ligand binding was carried out in 96 well plates (final volume 100 μL) with 2–5 μg membrane protein per well. Reaction mixtures were filtered over GF/C filters using a Brandel harvester to collect bound ligand. [^{35}S]GTPγS binding assays were performed in 5 mL Sarstedt tubes with 1 μg membrane protein per point in GTPγS binding buffer (50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM DTT, 1 μM GDP and 100 mM NaCl). Tubes containing