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Rapid communication

The role of G-proteins in the dimerisation of human somatostatin receptor types 2 and 5

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ABSTRACT

Somatostatin (SST) is a peptide hormone that acts through a family of heptahelical receptors belonging to the G-protein coupled receptor (GPCR) superfamily. There are five known SST receptor subtypes termed SSTR1–5 and all couple to $G\alpha_{i/o}$ G-proteins. It has been previously demonstrated that these receptors can form both homo- and heterodimers within their family or with other GPCR family members. Although agonist was demonstrated as a factor in modulating certain dimeric pairs, the molecular mechanism(s) underlying this regulation remains undetermined. Here, we demonstrate the coupling of G-protein as a contributing factor in the homo- and heterodimerisation of human (h) SSTR2 and SSTR5. When cells stably expressing hSSTR2 are pretreated with pertussis toxin (PTX), dissociation of hSSTR2 dimers occurs. Interestingly, although dimerisation of hSSTR5 was unaffected following PTX treatment, heterodimerisation between hSSTR2 and hSSTR5 is potentiated in the absence of receptor-stimulation. These results demonstrate the importance of G-protein in the maintenance and regulation of hSSTR dimers.

1. Introduction

Discovered over thirty years ago, somatostatin (SST) was originally identified in hypothalamic extracts as a negative regulator of growth hormone secretion from the anterior pituitary [1,2]. It has since been shown to modulate various cellular processes including neurotransmission, cell secretion and cell proliferation, through a family of cell surface receptors termed SSTRs [1,2]. There are five known SSTR subtypes (SSTR1–5) and all belong to the heptahelical G-protein coupled receptor superfamily (GPCR). Although the signalling elicited by SST varies depending on the SSTR subtype activated, all SSTRs are known to inhibit adenylyl cyclase by coupling to the pertussis toxin (PTX) sensitive $G\alpha_{i/o}$ class of G-proteins [1,2]. We and others have previously reported on the homo- and heterodimerisation of SSTRs within their family and amongst other GPCR family members [3–12]. Dimerisation of SSTRs leads to changes in ligand binding, cell signalling, receptor desensitization, receptor trafficking and cell growth [3–14]. Although agonist has been demonstrated as a factor governing the stability of certain SSTR dimeric pairs, the mechanism (s) underlying this regulation remains poorly understood. There have been recent indications that G-protein itself can regulate the interaction of GPCRs. For instance, the dimerisation of neuropeptide Y1 and Y2 receptors is altered when the coupling of G-protein to the receptor is interrupted [15,16]. Similar findings were reported for the μ/δ-opioid receptor heterodimer, as disrupting the interaction of $G\alpha_i$ by either PTX treatment or deleting regions of the receptor implicated in G-protein coupling abolishes heterodimerisation [17,18]. Notwithstanding, precoupling of GPCRs with their heterotrimeric G-proteins is suggested important for export-control from the ER to the Golgi hence, trafficking to the cell surface [19]. In light of these observations, we investigated the contribution of G-proteins in the homo- and heterodimerisation of human (h) SSTR2 and SSTR5, two subtypes that have been well characterized with respect to their dimerisation properties and are the primary targets in SST-analogue therapy [20,21]. Here, we demonstrate using both co-immunoprecipitation and photobleaching fluorescence resonance energy transfer (pbFRET) techniques, that hSSTR2 dimers dissociate into monomers following PTX treatment. Inactivation of G-proteins did not affect the dimerisation of hSSTR5 however; it did markedly increase the heterodimerisation of hSSTR2 and hSSTR5 in stably co-expressing cells. Taken together, we provide evidence on the importance of G-protein in the maintenance and formation of SSTR dimers.

2. Materials and methods

2.1. Materials and antibodies

The peptides SST-14 and [Leu(8)-D-Trp-22, Tyr-25]-SST-28 (LTT-SST-28) were purchased from Bachem, Torrance, CA. The non-peptide agonist L-779,976 was provided by Dr. S.P. Rohrer from Merck & Co [22]. Fluorescein-conjugated and rhodamine-conjugated mouse monoclonal antibodies against hemagglutinin (HA) (12CA5) were purchased from Roche Molecular Biochemicals, Mannheim, Germany.

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Rhodamine-conjugated and unconjugated anti-c-Myc monoclonal antibodies, mouse monoclonal unconjugated anti-HA antibody and pertussis toxin (PTX) were purchased from Sigma-Aldrich, Inc., St. Louis, MO. Protein A/G-agarose beads were purchased from Calbiochem, EMD Biosciences, Darmstadt, Germany.

2.2. Constructs and expressing cell lines

Stable transfections of CHO-K1 and HEK 293 cells expressing amino-terminally HA-tagged human SSTR2 (hSSTR2), HA-tagged human SSTR5 and c-Myc-tagged human SSTR5 (hSSTR5) or both receptor subtypes were prepared by Lipofectamine transfection reagent. Constructs expressing HA-tagged hSSTR2 and HA-tagged hSSTR5 were made in the pCDNA3.1/Neo vector (neomycin resistance) and c-Myc-tagged hSSTR5 in the pCDNA3.1/Hygro vector (hygromycin resistance) as previously described [3,6,7]. Clones were selected and maintained in Dulbecco's DMEM supplemented with 10% fetal bovine serum (FBS) and 700 μg/ml neomycin. Co-transfected cells were maintained in medium containing both 700 μg/ml of neomycin and 400 μg/ml of hygromycin as previously described [3,7]. Cells were grown in a 37 °C incubator with 5% CO_2.

2.3. GTP binding assay

The GTP-binding assay was performed by measuring the amount of [^{35}S]GTPγS (GE Healthcare, Waukesha, WI) bound to membranes from HEK 293 cells stably co-expressing SSTR2 and SSTR5. In 100 μl GTP assay buffer (20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, 10 μM GDP), 50 μg membrane protein and 10 pM [^{35}S]GTPγS were added in culture tubes with or without 10 nM agonist (SST-14 and L-779,976). The reaction was incubated in a 30 °C water bath shaking for 1 h. To inactivate $G\alpha_i$ G-proteins, membrane was pretreated with 0.5 μg activated pertussis toxin (PTX) (10 mM DTT, 10 μM ATP, PBS pH 7.4 for 30 min at 30 °C) for 1 h at 30 °C prior to GTP binding. The reaction was terminated by the addition of 1 ml ice-cold GTP assay buffer. Following centrifugation, membrane pellets were washed thrice in assay buffer before the addition of 7 ml scintillation fluid and counted using a LKB beta scintillation counter (LKB-Wallach).

2.4. PbFRET microscopy and immunocytochemistry

PbFRET experiments were performed on CHO-K1 and HEK 293 cells as previously described [3,6,7]. Briefly, the effective FRET efficiency (E) was calculated in terms of a percent, based on the photobleaching (pb) time constants of the donor taken in the absence $(D-A)$ and presence $(D+A)$ of acceptor according to $E=1-(\tau_{D-A}/\tau_{D+A})\times 100$. CHO-K1 or HEK 293 cells were seeded on glass coverslips for 24 h and treated with or without PTX (100 ng/ml) for 18 h in the absence of serum followed by stimulation with agonist where indicated for 10 min at 37 °C. The reaction was terminated by placing cells on ice and washing in Dulbecco's PBS. Cells were then fixed in 4% paraformaldehyde for 15 min before being processed for immunocytochemistry. Antibodies used were mouse monoclonal anti-HA conjugated to fluorescein and rhodamine as the donor and acceptor respectively. In case of SSTR2/SSTR5 co-expressing cells, pbFRET was performed with monoclonal anti-HA conjugated to fluorescein directed to SSTR2 as the donor and monoclonal anti-c-Myc conjugated to rhodamine directed to SSTR5 as the acceptor. A series of twenty images was captured using a Leica DMBL fluorescent microscope. Areas consisting of the plasma membrane were used for analyzing the photobleaching decay rates on a pixel-by-pixel basis.

2.5. Co-immunoprecipitation and Western blot

To inactivate $G\alpha_i$ G-proteins, membrane proteins (500 μg) from stably co-transfected HEK 293 cells were pretreated with 2.5 μg activated PTX (10 mM DTT, 10 μM ATP, PBS pH 7.4 for 30 min at 30 °C) for 1 h at 30 °C as previously described [3,23]. The reaction was terminated by the addition of 1 ml ice-cold PBS followed by treatment with 10 nM of agonist L-779,976 in binding buffer (50 mM HEPES, 2 mM $CaCl_2$, 5 mM $MgCl_2$, pH 7.5) for 30 min at 37 °C. Following treatment, membrane proteins were solubilised in 1 ml RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, pH 8.0) for 1 h at 4 °C. Samples were incubated with anti-HA antibody for immunoprecipitation and purified with Protein A/G-agarose beads. Purified proteins were fractionated by electrophoresis on a 7% SDS polyacrylamide gel and transferred to PVDF membrane (GE Healthcare, Piscataway, NJ), as previously described [3,6,7]. Immunoblotting of the heterodimer was performed using anti-c-Myc antibody (1:2000). Blocking of membrane, incubation of primary antibodies, incubation of secondary antibodies and detection by chemiluminescence were performed following ECL Western blotting detection kit (GE Healthcare, Piscataway, NJ) according to manufacturer's instructions. Images were captured using an Alpha Innotech FluorChem 8800 (Alpha Innotech Co., San Leandro, CA) gel box imager and densitometry was carried out using FluorChem software (Alpha Innotech Co.).

2.6. Statistical analysis

Data were analyzed by ANOVA, post hoc Dunnett's using Graph Pad Prism 4.0. Statistical differences were taken at P values <0.05. At least three independent experiments were performed for each statistical analysis.

3. Results

3.1. Dimerisation of hSSTR2 and hSSTR5 and G-proteins

CHO-K1 cells stably expressing hSSTR2 at expression levels of 435 ± 33 fmol/mg of protein [6], were left untreated or pretreated with PTX for 18 h. To determine the effectiveness of PTX on our stably transfected cells, we measured for changes in GTP binding. Pretreatment of hSSTR2 transfected cells with PTX effectively blocked GTP binding induced upon stimulation with either the SSTR2-specific agonist L-779,976 or SST-14 (Fig. 1). Cells were then stimulated with or without 100 nM SST-14 and measured for changes in dimerisation using the pbFRET microscopy technique. Under basal conditions in the absence of agonist (control),

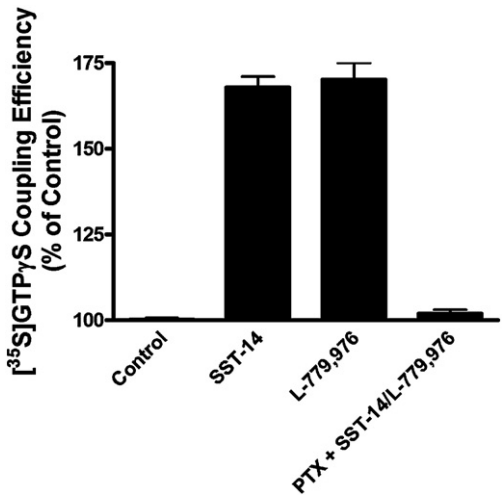

Fig. 1. Inhibition of GTP binding by PTX. GTP binding of membranes extracted from HEK 293 cells stably expressing SSTR2 were treated with 10 nM of either SST-14 or L-779,976 agonists where indicated. GTP binding was inhibited when membranes were pretreated with PTX. Means ± SEMs are representative of at least three independent experiments performed in duplicate.