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Homologous desensitization of human histamine H_3 receptors expressed in CHO-K1 cells

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ABSTRACT

Histamine H_3 receptors (H_3Rs) modulate the function of the nervous system at the pre- and post-synaptic levels. In this work we aimed to determine whether, as other G protein-coupled receptors (GPCRs), H_3Rs desensitize in response to agonist exposure. By using CHO-K1 cells stably transfected with the human H_3R (hH_3R) we show that functional responses (inhibition of forskolin-induced cAMP accumulation in intact cells and stimulation of [^{35}S]-GTPγS binding to cell membranes) were markedly reduced after agonist exposure. For cAMP accumulation assays the effect was significant at 60 min with a maximum at 90 min. Agonist exposure resulted in decreased binding sites for the radioligand [^{3}H]-N-methyl-histamine ([^{3}H]-NMHA) to intact cells and modified the sub-cellular distribution of H_3Rs, as detected by sucrose density gradients and [^{3}H]-NMHA binding to cell membranes, suggesting receptor internalization. The reduction in the inhibition of forskolin-stimulated cAMP formation observed after agonist pre-incubation was prevented by incubation in hypertonic medium or in ice-cold medium. Agonist-induced loss in binding sites was also prevented by hypertonic medium or incubation at 4 °C, but not by filipin III, indicating clathrin-dependent endocytosis. Immunodetection showed that CHO-K1 cells express GPCR kinases (GRKs) 2/3, and both the GRK general inhibitor $ZnCl_2$ and a small interfering RNA against GRK-2 reduced receptor desensitization. Taken together these results indicate that hH_3Rs experience homologous desensitization upon prolonged exposure to agonists, and that this process involves the action of GRK-2 and internalization via clathrin-coated vesicles.

1. Introduction

The primary function of the G protein-coupled receptors (GPCRs) in the nervous system is to act as mediators of the so-called slow chemical synaptic transmission (Gainetdinov et al., 2004), and in the mammalian brain histamine regulates several functions by interacting mainly with three (H_1, H_2 and H_3) of its four receptors cloned so far (H_1–H_4; Haas et al., 2008).

Histamine H_3 receptors (H_3Rs) are primarily located on nerve terminals where they control the release and synthesis of histamine as well as the release of several other neuroactive substances namely acetylcholine, noradrenaline, dopamine, γ-aminobutyric acid (GABA), glutamate, 5-hydroxytryptamine (5-HT) and substance P (Leurs et al., 2005; Feuerstein, 2008; Haas et al., 2008). However, there is also evidence for a post-synaptic expression of H_3Rs in some regions of the brain such as the striatum (projection neurons), cerebral cortex (layer V pyramidal neurons) and hippocampus (granule cells) (Pillot et al., 2002).

Through their coupling to Gαi/o proteins H_3Rs trigger several signaling pathways that include inhibition of adenylyl cyclase and thus of protein kinase A (PKA) activation, inhibition of voltage-operated Ca^{2+} channels thereby modulating neurotransmitter release, activation of phospholipase A_2 (PLA_2) leading to the release of arachidonic acid, modulation of the mitogen-activated protein kinase (MAPK) pathway and activation of the Akt/GSK-3β axis (Bongers et al., 2007a).

The functional response of GPCRs is regulated at the level of the receptor itself by two primary mechanisms: regulation of receptor

Abbreviations: H_3R, histamine H_3 receptor; GPCR, G protein-coupled receptor; GRK, GPCR kinase; RAMH, *R*-α-methylhistamine.

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density at cell surfaces and control of signaling efficiency (Hill, 2006). Homologous desensitization, an important regulatory mechanism that prevents GPCR overstimulation, implies a reduction in signaling upon agonist binding and receptor activation (Ferguson et al., 1996). The canonical mechanism involves phosphorylation of the occupied receptor by GPCR kinases (GRKs) resulting in increased affinity of the phosphorylated receptor for the proteins β-arrestins, which upon binding to the GPCR inhibit further coupling to G proteins and interact with clathrin and the β2-adaptin subunit of the clathrin adaptor AP-2 (adaptor protein complex-2) to target the receptor to clathrin-coated pits and the endocytic machinery, leading to receptor internalization (Gainetdinov et al., 2004). Endocyted receptors can be either re-inserted into the membrane or degraded in lysosomes, with the latter resulting in a reduction of total receptor number (down-regulation). The time-course of these processes spans from seconds (phosphorylation) to minutes (endocytosis) and hours (down-regulation) (Ferguson et al., 1996; Pierce et al., 2002).

The β-adrenoceptors and, to a lesser extent, muscarinic receptors are to date the best studied regarding their desensitization and the mechanisms involved. For histamine receptors there is evidence that both H_1 and H_2 receptors undergo desensitization. While GRK-2 appears responsible for the desensitization of endogenous H_1 receptors expressed by uterine smooth-muscle cells (Self et al., 2005; Willets et al., 2008), in COS-7 cells desensitization of transfected H_2 receptors is mediated by GRK-2 and GRK-3 (Shayo et al., 2001).

Some pieces of evidence suggest that H_3Rs also experience homologous desensitization. In guinea-pig ileum where H_3R activation inhibits electrically-induced contraction most likely by reducing acetylcholine release from postganglionic cholinergic neurons, previous exposure to the agonist R-α-methylhistamine (RAMH) results in a reduction in both agonist potency and efficacy in a second agonist application (Perez-Garcia et al., 1998; Alguacil and Perez-Garcia, 2003). Further, we previously reported that the incubation of rat striatal slices with H_3R agonists results in a significant decrease in receptor density, as assessed by the binding of the labeled agonist [^{3}H]-N-methyl-histamine ([^{3}H]-NMHA) to membranes of treated slices. This effect was prevented, although partially, by incubation in a hypertonic medium suggesting the participation of clathrin-mediated endocytosis (Garduno-Torres and Arias-Montano, 2006).

The H_3R has become a promising drug target for the treatment of neuropathic pain, sleep-wake disorders and cognitive impairment associated with Alzheimer's disease, attention deficit hyperactivity disorder (ADHD), schizophrenia and Parkinson's disease (Passani and Blandina, 2011). While H_3R antagonists/inverse agonists have attracted most attention, with pitolisant, GSK-189254 and JNJ-31001074 already in phase II-III clinical trials, H_3R agonists may be useful in the treatment of some types of insomnia (Passani et al., 2004), neuropathic pain (Cannon and Hough, 2005) and in the prevention of excitotoxic neuronal damage (Lintunen et al., 2005; Kukko-Lukjanov et al., 2006; Mariottini et al., 2009). One common drawback of otherwise effective pharmacological treatments is receptor desensitization, and in this work we therefore set out to study further whether H_3Rs experience homologous desensitization by using a heterologous expression system.

2. Materials and methods

2.1. Cloning of the hH_3R

The hH_3R of 445 amino acids (hH_3R_{445}) was amplified by PCR from the human male PAC clone RP5-1005F21 and subsequently cloned into the pCIneo expression vector. DNA sequencing confirmed the identity of the cloned hH_3R with the published hH_3R_{445} sequence (GenBank accession number NM_007232).

2.2. Construction of small-hairpin RNA (shRNA) expression vectors

In order to assess the effect of reducing endogenous GRK-2 (adrbk1) levels by RNA interference, shRNA-expression vectors were engineered to co-express shRNAs and the green fluorescent protein (GFP) which allowed for monitoring cell transfection. The plasmid psh-adrbk1-GFP was created by ligating the annealed oligonucleotide duplex (*sense*: 5′-GATCCGATGTGTTCCAGAAGTTCATTCAAGAG ATGAACTTCTGGAACACATCTTTTTACGCGTG-3′; *antisense*: 5′-AATTCACGCGTAAAA AGATGTGTTCCAGAAGTTCATCTCTTGAATGAACTTCTGGAACACATCG-3′) into the *Bam*HI and *Eco*RI sites in the U6 expression cassette of the pLVX-shRNA2 vector (Clontech, Mountain View, CA, USA). The resulting shRNAs targeted hamster *adrbk1* base pairs 353–372 (GenBank accession number XM_003509944). The non-silencing shRNA sequence (psh-scramble) was designed to have no homology with any mammalian gene. Efficacy and selectivity of psh-adrbk1-GFP were tested by determining protein expression by Western blotting with a human anti-ADRBK1 antibody (anti GRK-2, Santa Cruz Biotechnology, Dallas, TX, USA). A second shRNA that targeted the position 1061–1080 was also evaluated and showed similar efficacy. All three U6-driven shRNAs were confirmed by DNA sequencing.

2.3. Cell culture and transfection

CHO-K1 cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium/Nutrient F-12 mix (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic and antimycotic.

For stable hH_3R_{445} transfection, CHO-K1 cells were grown to 80–90% confluence and cDNA constructs were transfected using Lipofectamine 2000 (Invitrogen, Life Technologies, Grand Island, NY, USA) after protocol optimization as recommended by the supplier. Transfected cells were selected with 600 μg/ml geneticin (G-418), tested for receptor expression by radioligand binding and maintained in the presence of 400 μg/ml geneticin. Transient transfection with shRNA expression vectors was performed with Lipofectamine 2000, and experiments were carried out 48 h after transfection.

2.4. Desensitization experiments

Cells were incubated for the indicated times (90 min for most experiments) in Krebs–Ringer–Hepes (KRH) buffer or serum-free DMEM-F12 medium (for immunocytochemistry experiments) in the presence and the absence of the selective H_3R agonist RAMH (100 nM).

In order to wash out the agonist at the end of the incubation period the medium was removed, cells were rinsed three times with fresh medium pre-warmed at 37 °C and incubated for 5 min in the same medium. Cells were rinsed once more before performing the corresponding assay. The composition of the KRH medium was (in mM): NaCl 108, $NaHCO_3$ 25, KCl 3, $MgCl_2$ 1, KH_2PO_4 1, $CaCl_2$ 1.8, D-glucose 11, Hepes 20; pH 7.4 with NaOH.

2.5. [^{3}H]-N-methyl-histamine ([^{3}H]-NMHA) binding assays

2.5.1. [^{3}H]-NMHA binding to cell membranes

CHO-K1-hH_3R_{445} cells grown in plastic Petri dishes (100-mm diameter) were scrapped and homogenized in ice-cold Tris–HCl buffer (10 mM Tris–HCl, 1 mM EGTA, pH 7.4), and centrifuged (42,000 × *g* for 20 min at 4 °C). Pellets were re-suspended in incubation buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.4) and aliquots (50 μg protein) incubated (60 min, 30 °C) in 100 μl incubation buffer containing [^{3}H]-NMHA (1.5 nM for single-point determinations or 0.01–10 nM for saturation experiments). Non-specific binding was determined in the presence of 10 μM histamine. Incubations were stopped by rapid filtration through glass microfiber filters Whatman GF/B, presoaked in 0.3% polyethylenimine, and radioactivity trapped determined by scintillation counting. Protein contents were determined by the biccinchonic acid assay (BCA; Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as standard, and data used to estimate receptor binding as fmol/mg protein.

2.5.2. [^{3}H]-NMHA binding to intact cells

CHO-K1-hH_3R_{445} cells grown in 24-well plates (3×10^5 cells/well) were washed twice with KRH solution and pre-incubated for 90 min (37 °C) in the same solution in the presence and the absence of the H_3R agonist RAMH. Cells were rinsed as described above before incubation (5 h, 4 °C) in 250 μl KRH buffer containing [^{3}H]-NMHA (6 nM for single-point determinations or 0–10 nM for saturation experiments). Non-specific binding was determined in the presence of 10 μM histamine or thioperamide, with similar results. After incubation the medium was removed and cells were washed three times with 500 μl ice-cold KRH buffer before being lysed with 200 μl deionized H_2O containing Triton X-100 (1% v:v). Lysates were collected and each well was washed with 250 μl deionized H_2O, which were collected and added to the corresponding sample. Scintillation liquid was added to determine the radioactivity present in each sample. Receptor binding was expressed as sites per cell after cell counting.