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The internalization of the M_2 and M_4 muscarinic acetylcholine receptors involves distinct subsets of small G-proteins

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Abstract

Multiple mechanisms exist for the endocytosis of receptors from the cell surface. While the M_1, M_3, and M_4 subtypes of muscarinic acetylcholine receptors internalize through the well-characterized mechanism of clathrin coated vesicles, the mechanism of M_2 endocytosis is not well defined. Because the M_2 and M_4 receptors transduce their signals through the same second messengers but internalize though different pathways, we tested the ability of several small G-proteins to regulate the agonist-induced endocytosis of M_2 and M_4 in JEG-3 human choriocarcinoma cells. Dominant-negative Rab5 as well as both wild-type and dominant-negative Rab11 inhibited M_4 but not M_2 endocytosis. In contrast, a dominant-negative Arf6 as well as wild-type Rab22 increased M_2 but not M_4 endocytosis. We used immunocytochemistry to show that in unstimulated cells, the M_2 and M_4 receptors co-localize on the cell surface, whereas after stimulation M_2 and M_4 are in distinct vesicular compartments. In this study, we demonstrate that agonist-induced internalization of the M_2 receptor utilizes an Arf6, Rab22 dependent pathway, while the M_4 receptor undergoes agonist-induced internalization through a Rab5, Rab11 dependent pathway. Additionally, we show that Rab15 and RhoA are not involved in either pathway in JEG-3 cells.

Keywords: Muscarinic; Endocytosis; Small G-proteins; GPCR; Trafficking; Vesicle

Introduction

Muscarinic acetylcholine receptors (mAChRs) are members of the G-protein coupled receptor (GPCR) superfamily whose members couple with heterotrimeric G-proteins to transduce extracellular signals into intracellular signal transduction cascades (Lanzafame et al., 2003; van Koppen and Kaiser, 2003). The mAChR family consists of five subtypes (M_1–M_5) which can be divided into two groups based on the signal transduction pathway to which they couple most efficiently; M_1, M_3, and M_5 couple to the Gq family of G-proteins, while M_2 and M_4 couple to the Gi family (van Koppen and Kaiser, 2003).

Several mechanisms are responsible for regulating cell signal transduction following stimulation of a GPCR. In seconds to minutes after agonist exposure, desensitization, involving G-protein coupled receptor kinase (GRK) and β-arrestin, uncouples the receptor from its G-protein (Ferguson, 2001). Agonist-induced receptor endocytosis also occurs rapidly to remove receptors from the cell surface. Receptor endocytosis has several functions that depend on the specific receptor and cell type, including coupling receptors to different signal transduction cascades (Pierce et al., 2001), dephosphorylation and resensitization of receptor function (Claing et al., 2002), and initiating downregulation (von Zastrow, 2003) of the receptor. Additionally, internalization of one receptor can influence the signaling of an unrelated receptor, as endocytosis of the epidermal growth factor receptor contributes to the signal transduction of the adrenergic receptors (Pierce et al., 2000). Several mechanisms of receptor endocytosis have been characterized. The most well understood mechanisms are mediated by clathrin coated pits (Le Roy and Wrana, 2005), where a clathrin matrix assembles causing an invagination which is then pinched from the membrane in a dynamin-dependent fashion, and caveolae,

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which utilize caveolin and are sometimes associated with lipid rafts (Nichols, 2003; Parton and Richards, 2003). Perplexingly, several receptors have been shown to not internalize through either of these pathways, though the pathways that they utilize are not yet well-characterized. M_1, M_3, and M_4 mAChRs undergo agonist-induced endocytosis in a dynamin-dependent manner through clathrin coated pits, while M_2 displays a unique sensitivity to dynamin in HEK cells and does not utilize clathrin coated pits (Tolbert and Lameh, 1996; Schlador and Nathanson, 1997; Shockley et al., 1999; van Koppen, 2001; Roseberry and Hosey, 2001; Roseberry et al., 2001; Delaney et al., 2002). Some studies have shown an interaction between M_2 and caveolin in cardiomyocytes (Feron et al., 1997), although the internalization of the M_2 receptor in HEK293 cells has been reported to be independent of caveolae (Roseberry and Hosey, 2001).

We have previously shown that JEG-3 choriocarcinoma cells are an attractive system for the analysis of agonist-induced internalization of the M_2 receptor. Initial work showed that the M_2 but not the M_1 receptor undergoes agonist-induced internalization in these cells (Goldman et al., 1996). These observations provided the first evidence that multiple pathways exist for agonist-induced internalization of the mAChRs. In addition, while M_2 internalization can be increased by overexpression of GRK2 and β-arrestin-1 (Schlador and Nathanson, 1997), this is not the preferred endocytic pathway for M_2 as demonstrated by the observation that $M_2/M_1(6)$, an internalization deficient mutant M_2, is able to undergo endocytosis only when co-expressed with these proteins (Schlador et al., 2000). The differential internalization of M_1 and M_2 has allowed identification of five specific amino acid residues residing in the third intracellular loop as well as the sixth and seventh transmembrane domains required for this M_2-specific internalization pathway (Schlador et al., 2000). Like M_1, the M_3 receptor is also relatively resistant to agonist-induced internalization in JEG-3 cells (Goin and Nathanson, unpublished data), while the M_4 receptor exhibits internalization that is comparable in magnitude to M_2 (Schlador, 2000).

The Rab and Arf families of small G-proteins were originally discovered as GTPases involved in transport within the trans-Golgi network, but it has recently become clear that they are also involved in internalization of cell surface receptors. In fact, some members of these families are found on the plasma membrane and endosomes where their main function is in regulating vesicular transport (Takai et al., 2001). Arf6, Rab5, Rab11, and Rab4 have all been implicated in the endocytosis or recycling of the beta-adrenergic receptor following ligand stimulation (Premont et al., 1998; Seachrist et al., 2000; Moore et al., 2004). Additionally, Arf6 has been implicated in M_2 endocytosis (Delaney et al., 2002; Houndolo et al., 2005) while Rab5 and Rab11 have been implicated in M_4 endocytosis and recycling, respectively (Volpicelli et al., 2001; Volpicelli et al., 2002). To date, there has not been a systematic comparison of the regulation of different muscarinic receptor subtypes by small G-proteins. Therefore, we sought to determine whether the endocytic pathways of M_2 and M_4, which are highly homologous receptors with similar signal transduction properties, are completely distinct or if their pathways involve some shared modulators. We demonstrate here that the M_2 endocytic pathway utilizes Arf6 and Rab22 while M_4 uses Rab5 and Rab11 for its endocytic pathway. We further demonstrate that M_2 and M_4 do not co-localize in vesicles during their initial stages of endocytosis.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin–streptomycin (P/S) were obtained from Life Technologies. Lipofectamine2000 was from Invitrogen. N-[^{3}H]methylscopolamine ([^{3}H]NMS, 80–82 Ci/mmol) was purchased from Amersham. The chamber slides were from Nalge Nunc International. The anti-HA rabbit polyclonal antibody was from Pierce. The Alexa 568 conjugated anti-mouse antibody and the Alexa 488 conjugated anti-rabbit antibody were from Molecular Probes. The anti-Flag mouse monoclonal M2 antibody, carbamylcholine chloride (carbachol), atropine and all other reagents were purchased from Sigma.

Plasmids

The Flag-M_2 pcDNA3.1 construct was generated by digesting pCDPS-Flag-M_2 (Schlador and Nathanson, 1997) with Kpn1 and EcoR1 to remove the Flag-M_2 coding region, which was then ligated into the pCDNA3.1 vector (Invitrogen). The HA-M_4, HA-RhoA, HA-RhoAS19N, and HA-RhoAG14V constructs were purchased from the Guthrie (now the UMR) cDNA Resource Center. Rab5-GFP and Rab5S34N-GFP were the generous gift of Dr. Nigel Bunnett (UCSF) (Schmidlin et al., 2001). Dr. Aimee Powelka kindly provided the Arf6, Arf6T27N, and Arf6Q67L constructs (Powelka and Buckley, 2001). Dr. Ann Richmond (Vanderbilt University School of Medicine, Nashville, TN) kindly provided the Rab11 and Rab11S25N constructs (Fan et al., 2003). Rab15, Rab15N121I, Rab15T22N, and Rab5Q67L constructs were the generous gift of Dr. Lisa Elferink (University of Texas Medical Branch, Galveston, TX) (Zuk and Elferink, 1999). The Rab22, Rab22S19N, and Rab22Q64L constructs were the generous gift of Dr. Luis Mayorga (Universidad Nacional de Cuyo, Argentina) (Mesa et al., 2001).

Cell culture and transfection

JEG-3 choriocarcinoma cells (American Type Culture Collection, Rockville, MD) were grown in DMEM supplemented with 10% FBS and 1% P/S in a 10% CO_2 environment at 37 °C. For assays, a 10 cm plate was transected using Lipofectamine2000 with 6–10 μg DNA each of both receptor and G-protein expression vectors.

Binding assays

Cell surface expression of mAChRs was measured using the binding of the membrane-impermeable radioligand N-[^{3}H]