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Novel Interaction between the M_4 Muscarinic Acetylcholine Receptor and Elongation Factor 1A2*

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The activation of the muscarinic acetylcholine receptor (mAChR) family, consisting of five subtypes (M_1–M_5), produces a variety of physiological effects throughout the central nervous system. However, the role of each individual subtype remains poorly understood. To further elucidate signal transduction pathways for specific subtypes, we used the most divergent portion of the subtypes, the intracellular third (i3) loop, as bait to identify interacting proteins. Using a brain pull-down assay, we identify elongation factor 1A2 (eEF1A2) as a specific binding partner to the i3 loop of M_4, and not to M_1 or M_2. In addition, we demonstrate a direct interaction between these proteins. In the rat striatum, the M_4 mAChR colocalizes with eEF1A2 in the soma and neuropil. In PC12 cells, endogenous eEF1A2 co-immunoprecipitates with the endogenous M_4 mAChR, but not with the endogenous M_1 mAChR. In our *in vitro* model, M_4 dramatically accelerates nucleotide exchange of eEF1A2, a GTP-binding protein. This indicates the M_4 mAChR is a guanine exchange factor for eEF1A2. eEF1A2 is an essential GTP-binding protein for protein synthesis. Thus, our data suggest a novel role for M_4 in the regulation of protein synthesis through its interaction with eEF1A2.

In the central nervous system, the muscarinic acetylcholine receptors (mAChR)[1] play crucial roles in learning, memory, movement, analgesia, and sleep (1–3). Dysfunction in mAChR signaling has been implicated in brain disorders, including Alzheimer's disease, Parkinson's disease, and schizophrenia (4–6). The mAChRs belong to the G-protein-coupled receptor (GPCR) superfamily. Upon agonist binding, GPCRs bind and activate heterotrimeric G-proteins, which in turn activate various downstream targets. There are two distinct, well characterized G-protein signaling pathways activated by the five mAChR subtypes. M_1, M_3, and M_5 couple to G_q, which stimulates phospholipase C-β, and thus releases calcium from intracellular stores and activates protein kinase C (7, 8). M_2 and M_4 are coupled to $G_{i/o}$, which regulates adenylate cyclase (9). These subtypes are expressed throughout the brain, and, in some brain regions, multiple subtypes are expressed in individual neurons (10). The diversity of physiological effects and the overlapping expression pattern of the muscarinic receptor subtypes suggest that there are signaling pathways initiated by the mAChRs independent of these two heterotrimeric G-protein pathways.

Signaling pathways independent of heterotrimeric G-proteins have been reported throughout the GPCR superfamily (11–14). Evidence supporting this hypothesis stems from the identification of novel binding partners of GPCRs other than the traditional heterotrimeric G-proteins. Within the muscarinic family in particular, there is an especially large body of evidence to suggest the existence of nontraditional signaling pathways. There are many reports of G-protein-independent regulation of ion channels by mAChRs (15–18). Moreover, the M_3 mAChR has been found to associate with ADP ribosylation factor and Rho in an agonist-dependent manner. Furthermore, inhibition of ADP ribosylation factor and Rho abolished muscarinic activation of phospholipase D, whereas inhibition of heterotrimeric G-proteins had no effect on the activation of phospholipase D (19). Given the diversity of mAChRs, it is likely that other signaling pathways remain to be discovered.

We set out to identify novel binding partners of the mAChR subtypes to further elucidate the molecular events involved in mAChR signaling in the central nervous system. GPCRs consist of seven transmembrane domains connected by three extracellular loops and three intracellular loops. The intracellular third (i3) loop connecting the fifth and sixth transmembrane domains is an important signaling structure in the GPCR superfamily providing key sites of interactions with the heterotrimeric G-proteins, G-protein receptor kinases, and arrestins (20–22). The i3 loops of the mAChR are some of the largest in the GPCR superfamily (156–239 amino acids) and contain no homology between the mAChR subtypes except for ~20 amino acids at the N and C termini. We used the most divergent portion of the i3 loops as bait to affinity isolate and identify brain proteins that are novel binding partners of the mAChR subtypes. We have found a novel interaction between the M_4 mAChR and elongation factor 1A2 (eEF1A2) both *in vitro* and in cells with endogenous proteins. eEF1A is a GTP-binding protein that is essential in protein synthesis mediating the binding of the aminoacyl-tRNA to the acceptor site of the ribosome. The eEF1A2 isoform is only expressed in skeletal muscle, heart muscle, and brain in adult mammals (23, 26). We

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[1] The abbreviations used are: mAChR, muscarinic acetylcholine receptor; GPCR, G-protein-coupled receptor; GST, glutathione *S*-transferase; eEF, elongation factor; PBS, phosphate-buffered saline; GEF, guanine exchange factor; mantGDP, *N*-methylanthraniloyl-GDP; RP-HPLC, reverse phase-high performance liquid chromatography; NRS, normal rat serum; α-CAMKII, α subunit of calmodulin kinase II.

demonstrate that M_4 regulates the guanine nucleotide binding of eEF1A2, which has the potential to affect the role of eEF1A2 role in translation or other cellular processes.

MATERIALS AND METHODS

All reagents were purchased from Sigma unless noted otherwise.

Expression Plasmids—Segments of the human muscarinic receptor genes of M_1, M_2, and M_4 corresponding to the most divergent portions of the intracellular third (i3) loop (126, 135, and 152 amino acids, respectively) were subcloned into the bacterial expression vector pGEX2T as previously described (10). The human eEF1A2 and eEF1Bα were subcloned into the pET30a vector.

Induction and Purification of Fusion Proteins—The mAChR glutathione *S*-transferase (GST) fusion proteins (M_1i3-GST, M_2i3-GST, and M_4i3-GST) were induced and purified following the protocol as previously described except for the following modifications (10). The bacterial cultures were induced for 2 h with 500 μM isopropyl-β-D-thiogalactopyranoside. One-liter cultures were pelleted, and the cell pellets were resuspended in 25 ml of harvest buffer (10 mM HEPES, pH 7.3, 150 mM NaCl, 1 mM benzamidine, and 5 mM EDTA) with 1 mM lysozyme. The pellets then were frozen at −80 °C overnight. The samples were thawed and sonicated to remove any aggregated material. The solubilized proteins were obtained by two rounds of centrifugation each for 20 min at 15,000 × *g* at 4 °C. The fusion proteins were incubated with the glutathione-linked agarose beads for 1 h at 4 °C, and then washed five times with harvest buffer for purification. Soluble protein from 1 liter of bacteria was incubated with 100 mg of glutathione-agarose beads. eEF1Bα-His and eEF1A2-His were induced following the protocol for the GST fusion proteins except for the following modifications. eEF1A2-His was induced overnight at room temperature with 30 μM isopropyl-β-D-thiogalactopyranoside. Harvest buffer did not contain EDTA, and 10% glycerol was added to the harvest buffer for eEF1A2. eEF1A2-His and eEF1Bα-His were purified according to the QIAexpress system (Qiagen) using nickel-nitrilotriacetic acid-agarose (Qiagen). All fusion proteins were stored at −80 °C.

Brain Pull-down Assay—One rat brain (Pel-Freez) was homogenized and incubated at 4 °C in 30 ml of harvest buffer with 0.5% Triton X-100. The homogenate was centrifuged (15,000 × *g*) twice at 4 °C, and then precleared twice with GST adsorbed to 100 mg of agarose beads for 1 h at 4 °C. Next, mAChR i3 loop-GST fusion protein adsorbed to 100 mg of glutathione (GSH)-agarose beads was incubated with the 100 mg (6–10 mg/ml) of precleared brain homogenate for 1 h at 4 °C. The beads were pelleted at 1000 × *g* for 1 min at 4 °C and washed five times with 25 ml of harvest buffer. Finally, the beads were incubated with 2% SDS loading buffer for 15 min at 37 °C to elute adsorbed proteins from the bead matrix. Proteins were resolved on 4–20% SDS-PAGE (Novex) and visualized by staining the gel with Coomassie Blue.

In Vitro Binding Assay—GST, M_2i3-GST, or M_4i3-GST bound to 20 μl of glutathione-agarose beads were incubated with 80 μg of eEF1A2 (0.8 μg/μl) for 1 h at 4 °C in an Eppendorf tube with harvest buffer (without EDTA) and 30 μl of 10% bovine serum albumin. The total volume of the reaction was 1 ml. The beads were pelleted at 1000 × *g* for 1min at 4 °C and washed three times with harvest buffer without EDTA. Finally, the beads were incubated with 2% SDS loading buffer for 15 min at 37 °C to elute adsorbed proteins from the bead matrix, and the proteins were resolved on a 12% SDS-PAGE gel.

Immunoprecipitation—PC12 cells were maintained as previously described (24). Muscarinic subtypes were immunoprecipitated from PC12 cells as previously described (10, 24). Briefly, PC12 cells were homogenized with the Brinkman Polytron 3000 tissue grinder in TE (10 mM Tris, pH 7.4, 1 mM EDTA) and centrifuged at 15,000 × *g* for 3 min at 4 °C to isolate the membranes. The muscarinic receptors were solubilized at 1 mg/ml in TE containing 0.4% digitonin (Waco) and 0.08% cholate for 1 h at 4 °C, and centrifuged at 15,000 × *g* for 30 min at 4 °C. Each ml of solubilized material (1 mg/ml) was precleared with 50 μl of protein A beads at 4 °C overnight followed by preclearing for 6 h with 50 μl of protein A beads preincubated in normal rat serum (NRS). Immunoprecipitation reaction consisted of 750 μl of precleared soluble PC12 extract, 200 μl of 10% bovine serum albumin, and 5 μl of protein A beads preincubated overnight with M_1 or M_4 receptor crude rabbit antiserum. The immunoprecipitation was incubated for 4 h at 4 °C. The protein A beads were centrifuged at 4 °C for 1 min at 1000 × *g*, and pellets were washed five times with 0.1% TEDC (10 mM Tris, pH 7.4, 1 mM EDTA, 0.1% digitonin (Waco), 0.02% cholic acid) with 150 mM NaCl. Finally, the beads were incubated with 2% SDS loading buffer for 15 min at 37 °C to elute adsorbed proteins from the beads, and the proteins were resolved on a 12% SDS-PAGE. In some experiments, 100 μM carbachol (muscarinic agonist) or 10 μM atropine (muscarinic antagonist) were applied prior to the PC12 cells being harvested.

Western Blot—Samples were prepared in 2% SDS, separated by 12% SDS-PAGE, and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore). Membranes were processed as previously described except for the following modifications (24). Membranes were probed with mouse monoclonal eEF1A antibody (1:1000, Upstate Biotechnology), mouse monoclonal antibody to α subunit of calmodulin kinase II (α-CAMKII) (1:1000, Santa Cruz), or a mouse monoclonal GST antibody (1:5000, Bio-Rad). For some experiments, membranes were stripped by incubating them at 80 °C for 30 min in stripping buffer (62.5 mM Tris, pH 6.7, 100 mM β-mercaptoethanol, 2% SDS), and then washed to remove any stripping buffer with 0.2 M Tris-buffered saline before reprobing the membrane.

Immunocytochemistry—Sprague-Dawley rats were sedated with sodium pentobarbital and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer and post-fixed in 4% paraformaldehyde overnight. 50-μm sections were cut on a Vibratome. Sections were stored at −20 °C in 30% sucrose and 30% ethylene glycol.

All solutions were diluted in phosphate-buffered saline (PBS). The sections were rinsed in PBS and treated with 3% hydrogen peroxide for 10 min. After rinsing, the sections were blocked in 5% normal horse serum and 10 μg/ml avidin for 60 min at room temperature with gentle agitation. Primary antibody incubations were in buffer containing 1% normal horse serum, 50 μg/ml biotin, and 3 mM sodium azide. The primary antibodies used were M_4 monoclonal (1:250) and eEF1A (1:1000). For double labeling, both primaries were incubated together. The sections were rinsed and incubated for 60 min at room temperature with donkey anti-goat rhodamine X (1:100, Jackson Immunoresearch) in secondary buffer (1% normal horse serum). The sections were rinsed and incubated with biotinylated donkey anti-mouse secondary antibody (1:100, Jackson) in secondary buffer for 60 min at room temperature. The sections were rinsed and incubated in avidin-biotin complex (Vector) for 30 min, rinsed, and incubated in tyramide-fluorescein diluted in amplification diluent (1:100, PerkinElmer) for 10 min. The sections were rinsed and incubated for 30 min in 10 mM cupric sulfate in 50 mM ammonium acetate, pH 5.0, to eliminate autofluorescence. The sections were rinsed in PBS and mounted using Vectashield mounting media for fluorescence (Vector Laboratories). Control incubations included omission of primary antibodies to test nonspecific secondary antibody binding and incubation with one primary but both secondary antibodies to demonstrate the absence of bleed-through and cross-labeling (data not shown). Sections were scanned using a Zeiss LSM 510 laser scanning confocal microscope coupled to a Zeiss 100M Axiovert and a 63× Plan-Apochromat oil immersion lens. Adobe Photoshop was used for final image preparation.

Fluorescence N-methylanthraniloyl-GDP (mantGDP) Assays—eEF1A2-His was diluted in Buffer A (150 mM NaCl, 10 mM HEPES, pH 7.3, and 10% glycerol), and other fusion proteins were diluted in Buffer A without glycerol. MantGDP (Molecular Probes) was diluted in nucleotide buffer (10 mM HEPES, pH 7.3, 150 mM NaCl, 0.1 mg/ml bovine serum albumin, 10 mM $MgCl_2$, 0.2 mM diothiothreitol), and unlabeled nucleotides were diluted in deionized water. Experiments were performed on 96-well black plates (Costar) on ice, and the total volume per well was 200 μl. For each experiment, one set of wells contained mantGDP plus buffer A (background) and a parallel set of wells contained mantGDP plus eEF1A2-His. Fluorescence was determined by subtracting these two sets of wells. In experiments using additional fusion proteins and unlabeled nucleotides, the background well contained mantGDP plus these additions. The plates were read at room temperature with the Fmax Microplate Reader (Molecular Devices) and analyzed using the SOFTmax program. The plates were excited at 355 nm and read at 460 nm.

$$\text{Fluorescence}(F) = F[\text{mantGDP} + \text{eEF1A2} + (\text{variables})] - F[\text{mantGDP} + (\text{variables})] \quad \text{(Eq. 1)}$$

RESULTS

Identification of Proteins That Interact with the i3 Loop of mAChR Subtypes—To identify binding partners of the mAChRs, we used a pull-down assay that has been used to identify binding partners of other GPCRs (20, 25). GST fusion proteins of the i3 loop, excluding the conserved N and C termini, of the M_1, M_2, and M_4 subtypes (M_1i3-GST, M_2i3-GST, and M_4i3-GST, respectively) were expressed individually in bacteria and purified by binding to glutathione-agarose beads.

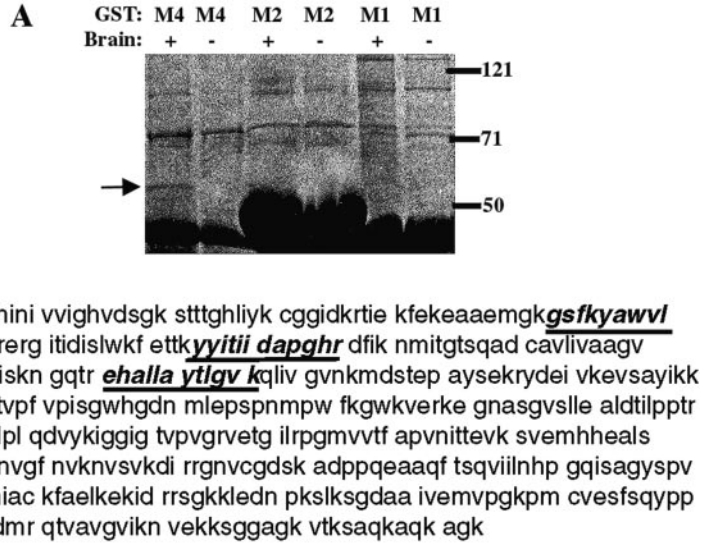

FIG. 1. **Identification of a protein that interacts with the M_4i3 loop.** Fusion proteins of the i3 loops of M_1, M_2, and M_4, or GST alone adsorbed to glutathione-agarose beads were individually incubated in brain homogenate. The beads were collected, washed, and placed in SDS loading buffer to elute any proteins off the beads. Protein eluates were separated on a 4–20% SDS-PAGE and stained with Coomassie Blue. *A*, a 52-kDa band (*arrow*) was specifically pulled down with the M_4i3-GST, but was not pulled down with the i3 loops of M_1 or M_2. *B*, the M_4i3-interacting protein band was subjected to in-gel tryptic digestion, fractionated by RP-HPLC, and sequenced by Edman degradation. The sequence of three peptides was determined by a PROWL search to be three different fragments of eEF1A2 (*underlined* amino acids).

Each fusion protein-bead mixture was individually incubated in brain homogenate. After the beads were collected and washed, the bound proteins were eluted and fractionated by SDS-PAGE, and then Coomassie Blue staining was used to identify proteins interacting with the mAChR i3 loops. A band at ~52 kDa was visualized in the brain pull-downs using the M_4i3-GST (Fig. 1*A*). This band was not a bacterial protein from the purification of the M_4i3-GST, because it was only present when the M_4i3-GST was incubated with the brain extract. The 52-kDa band interacted selectively with the M_4i3-GST, as it was not detected with the pull-down assays using M_1i3-GST, M_2i3-GST, or GST alone. Similar results were obtained in multiple independent pull-down experiments. To identify the 52-kDa protein, the band was sequenced after purification from brain using M_4i3-GST pull-down assay, in-gel tryptic digestion, and fractionation by RP-HPLC. Four peptides were recovered and sequenced by Edman degradation at the Microchemical Facility at the Emory University. A PROWL search of the rodent SWISS-PRO data base with the recovered peptides showed three of them to be within the sequence of protein eEF1A2 (Fig. 1*B*) and the fourth to be within α-CAMK-II. Both proteins have approximate molecular masses of 52 kDa.

To verify the sequencing results using independent methods, we probed the M_4i3-GST brain pull-down with α-CAMKII and eEF1A2 antibodies. The specificity of the interaction was assessed using the M_2i3-GST, because this is the most closely related subtype to M_4, and it shares coupling preferences to the $G_{i/o}$ proteins. All the pull-downs including GST alone contained immunoreactivity with the α-CAMKII antibody (data not shown), suggesting this was a nonspecific interaction with GST or agarose beads. In contrast, only the M_4i3-GST brain pull-down showed a 52-kDa band that was immunoreactive with the eEF1A2 antibody (Fig. 2*A*). Consistent with our initial brain pull-downs, there was no detectable eEF1A2 immunoreactivity present in the M_2i3-GST and GST brain pull-downs, even with excess levels of M_2i3-GST and GST. Hence, these results confirm that endogenous eEF1A2 selectively interacts with the M_4i3 loop.

Direct Interaction between M_4i3 Loop and eEF1A2—The results of the brain pull-down assay are consistent with either a direct interaction between M_4i3 loop and eEF1A2 or an indirect interaction involving other brain proteins. To determine whether the M_4i3 loop and eEF1A2 directly interact, we performed an *in vitro* binding assay using purified recombinant proteins (Fig. 2*B*). Recombinant eEF1A2 tagged with six histidines (eEF1A2-His) was individually incubated with M_4i3-GST, M_2i3-GST, or GST alone adsorbed to glutathione beads. The GST fusion proteins were collected and washed, and the eluted protein was analyzed by Western blot. eEF1A2 immunoreactivity was detected in the M_4i3-GST eluates, but not in eluates using M_2i3-GST or GST (Fig. 2*B*). Thus, the M_4i3 loop and eEF1A2 are capable of binding directly without the presence of other proteins. Moreover, post-translational modifications are probably not required for the interaction, because the recombinant proteins are unlikely to be post-translationally modified to the same extent in bacteria as they are in mammalian cells.

Endogenous M_4 mAChR Interacts with Endogenous eEF1A2—To begin to assess the biological relevance of this interaction, we sought to determine whether there is an interaction between the endogenous M_4 mAChR and eEF1A2 proteins in cells. First, we used immunohistochemistry to determine whether M_4 and eEF1A2 colocalize in the same neuronal population. We examined the rat striatum, where M_4 is expressed in a subset of medium spiny neurons (10). Consistent with previous reports using immunohistochemistry in mouse brain (27), eEF1A2 expression was found throughout the striatum (Fig. 3). Colocalization studies revealed that M_4 was expressed in a subset of eEF1A2-expressing cells. Both proteins were localized principally in the cell bodies, with no significant immunoreactivity in the nuclei. Colocalization of the proteins was also detected in dendrites, where M_4 is highly localized at postsynaptic sites, and where eEF1A is also expressed (28, 29). All neurons expressing M_4 also expressed eEF1A2. Thus, a population of neurons in the striatum has overlapping expression patterns of M_4 and eEF1A2 that would provide an opportunity for this interaction to occur *in vivo*.

We also evaluated the neurotypic PC12 cell line for colocalization of the endogenous proteins in a simpler system more amenable to biochemical analysis. As in the striatum, the proteins were colocalized in the PC12 cells using immunocytochemistry (data not shown). M_4 distribution was primarily at the cell surface in unstimulated cells and intracellular in a discrete punctae in agonist-treated cells as previously described (30). eEF1A2 immunoreactivity was found throughout the cell including at the plasma membrane, but no immunoreactivity was found in the nucleus. This staining pattern is similar as been reported in cultured human fibroblast cells using a different eEF1A antibody (37). This suggests there are numerous sites in intact PC12 cells where M4 and eEF1A2 could interact. To determine whether these two endogenous proteins physically interact in PC12 cells, we performed immu-

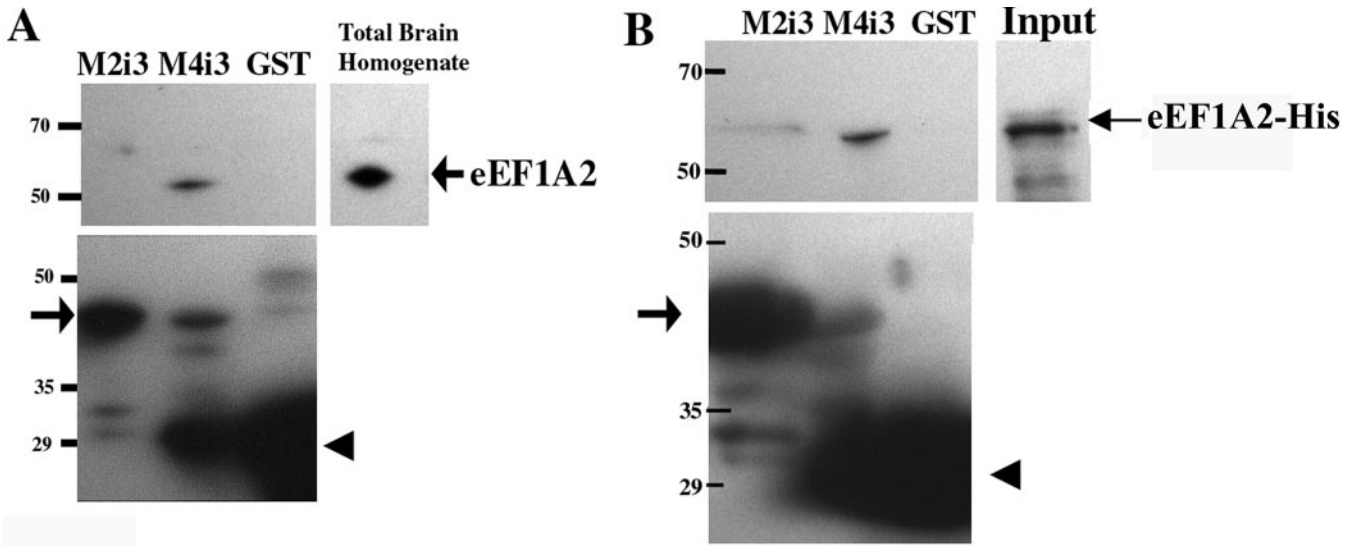

FIG. 2. **Specific and direct interaction of eEF1A2 with the M_4i3 loop.** *A*, using Western blot analysis, the brain pull-down assays were analyzed for eEF1A2 immunoreactivity. The M_4i3-GST pulled down eEF1A2 from solubilized rat brain, but not the M_2i3-GST or GST alone. 10 μg of brain homogenate that used for the pull-down was loaded for the total brain homogenate. *B*, eEF1A2-His was incubated with the M_4i3-GST, M_2i3-GST, or GST alone. M_4i3-GST binds eEF1A2 *in vitro*, but the M_2i3-GST or GST alone does not bind eEF1A2 *in vitro*. 2 μg of eEF1A2-His was loaded in the *Input lane*. The upper blots in *A* and *B* were incubated with an eEF1A2 antibody. For the loading control, the upper blots were stripped and incubated with a GST antibody to determine amount of protein loaded. The *arrow* marks the molecular weight for the full-length M_2i3-GST and M_4i3-GST; *arrowhead* marks the molecular weight for full-length GST.

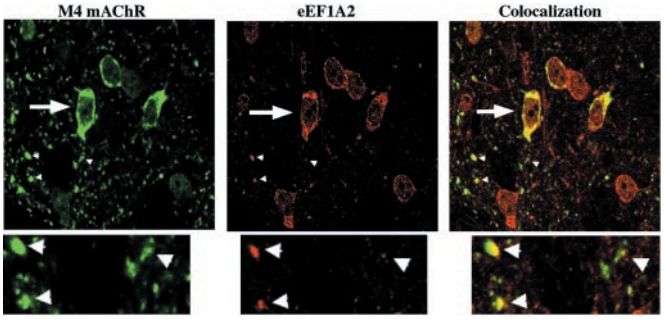

FIG. 3. **Co-localization of M_4 mAChR and eEF1A2 in brain.** Using confocal microscopy in the rat striatum, a subset of neurons (*arrow*) express both the M_4 mAChR (*green*) and eEF1A2 (*red*). In the soma, these proteins colocalize (*yellow*). *Yellow* punctae are also present outside the soma in the neuropil (*arrowhead*).

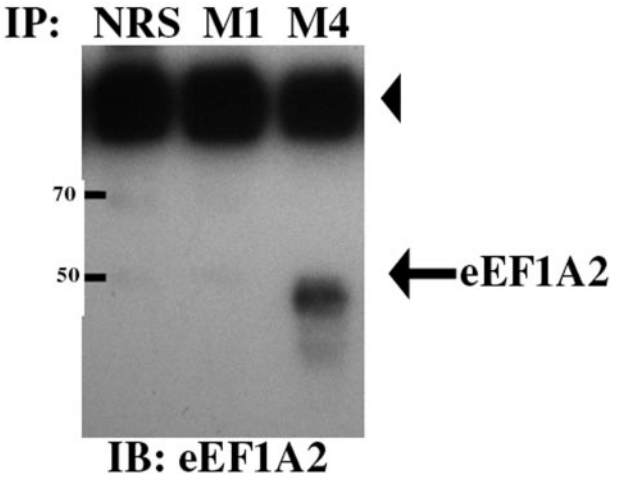

FIG. 4. **Endogenous M_4 mAChR associates with endogenous eEF1A2 in cells.** Soluble extract of PC12 cells was immunoprecipitated with M_1 rabbit antiserum, M_4 rabbit antiserum, or NRS. The immunoprecipitates were probed with an eEF1A2 antibody. eEF1A2 immunoprecipitated with endogenous M_4 mAChR, but not with endogenous M_1 mAChR or the NRS control. *Arrowhead* represents a nonspecific band. The figure is a representative blot of three separate immunoprecipitation experiments.

noprecipitations. The cells also express the M_1 mAChR, which was used as a control. The mAChR proteins were solubilized, and the receptor subtypes selectively immunoprecipitated with either M_1 rabbit antiserum, M_4 rabbit antiserum, or NRS as previously described (10, 24). One set of immunoprecipitates was radiolabeled with the muscarinic antagonist *N*-[^{3}H]methylscopalomine, confirming the presence of the mAChR (data not shown). A parallel set of immunoprecipitates was analyzed by Western blotting for the presence of eEF1A2 immunoreactivity. eEF1A2 co-immunoprecipitated with M_4, but was not found in the immunoprecipitates of M_1 or NRS (Fig. 4). Application of 100 μM carbachol (muscarinic agonist) or 10 μM atropine (muscarinic antagonist) at various time points (5, 15, 30, 45, 60, and 180 min) to the PC12 cells had no effect on the ability of M_4 to co-immunoprecipitate eEF1A2. These findings indicate that an interaction exists between endogenous M_4 and eEF1A2 in PC12 cells.

The M_4i3 Loop Behaves as a Guanine Exchange Factor for eEF1A2—To gain insight into the physiological relevance of this novel interaction, we explored the possibility that M_4 regulates eEF1A2 function. Upon activation, the mAChR i3 loop activates heterotrimeric G-proteins by acting as a guanine exchange factor (GEF), which stimulates the release of GDP allowing GTP to bind (31, 32). Hence, we hypothesized that M_4 may activate eEF1A2 similarly by regulating the nucleotide exchange rate of eEF1A2. To test this hypothesis, we measured the effects of the M_4i3 loop on the rate of GDP release using the fluorescence GDP analog mantGDP. The binding of mantGDP to a GTP-binding protein results in a change in fluorescence of the mant fluorophore. eEF1A2, unlike other GTP-binding proteins, has a very low affinity (0.01–3 μM) for guanine nucleotides (33). An advantage of mantGDP over radioligand binding assays is that it is performed in solution, and there is no need to separate bound and free ligand, which allows the detection of low affinity interactions.

First, we determined that eEF1A2-His was capable of binding guanine nucleotides. Increasing eEF1A2-His concentrations increased the fluorescence emitted by mantGDP (Fig. 5*A*).

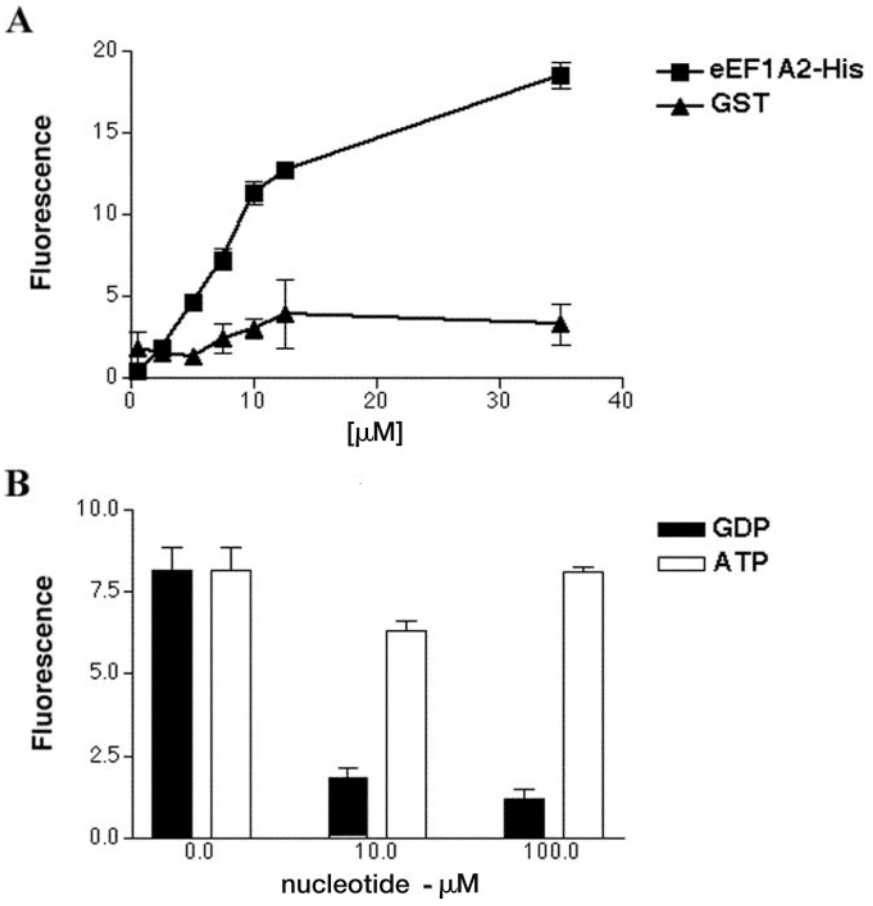

FIG. 5. **Nucleotide binding of eEF1A2-His.** *A*, increasing amounts of eEF1A2-His or GST (in triplicate) were added to 1 μM mantGDP. Increasing [eEF1A2-His] increased the emission of 1 μM mantGDP, whereas increasing [GST] had little effect. *B*, an excess of unlabeled nucleotides were added to eEF1A2-His before the addition of 1 μM mantGDP. Unlabeled GDP prevents mantGDP from binding eEF1A2-His, but unlabeled ATP has no effect. Each condition was performed in triplicate. Fluorescence (F) = F[mantGDP + eEF1A2-His] − F[mantGDP (+ GDP or ATP)].

In contrast, increasing concentrations of other proteins, such as GST, did not produce significant changes in the fluorescence of mantGDP, indicating the increase in fluorescence induced by eEF1A2-His was specific. To confirm the specificity of nucleotide binding to eEF1A2-His, we preincubated eEF1A2-His with excess unlabeled GDP or unlabeled ATP. Because eEF1A2 is a guanine nucleotide binding protein, the mantGDP binding should be sensitive to excess unlabeled GDP and not to excess ATP. As expected, excess GDP quenched the increased mantGDP fluorescence induced by eEF1A2, whereas ATP had no effect (Fig. 5*B*). Similar to experiments reported with the endogenous protein (34, 35), eEF1A2-His requires glycerol for maximum nucleotide binding (data not shown). Hence, these experiments validate that eEF1A2-His binds guanine nucleotides similarly to the endogenous protein and validate the mantGDP assay for monitoring the effects of protein interactions on eEF1A2 nucleotide exchange.

Next, we tested the hypothesis that the M_4i3 loop behaves as a GEF for eEF1A2 by promoting the release of mantGDP from eEF1A2. We measured the release of mantGDP from eEF1A2-His by the decrease in fluorescence upon the addition of excess unlabeled GDP. When excess unlabeled GDP is added to mantGDP-eEF1A2-His, there is ~20% decrease in fluorescence (Fig. 6). This represents a small percentage of the eEF1A2-His exchanging the mantGDP for unlabeled GDP. As a positive control, we examined the effect of a known eEF1A GEF, elongation factor 1Bα (eEF1Bα). eEF1Bα tagged with six histidines (eEF1Bα-His) decreased the fluorescence ~ 80% in the presence of excess unlabeled GDP. This demonstrates the ability of eEF1Bα to accelerate the nucleotide exchange of eEF1A as consistent with previous reports (35, 36). The M_4i3-GST had an effect similar to eEF1Bα-His, decreasing the fluorescence by ~50%, whereas GST had no effect. Thus, these results demonstrate that the M_4i3 loop behaves as an eEF1A2 GEF.

DISCUSSION

Using the i3 loop of M_1, M_2, and M_4 mAChR subtypes as bait, we discovered a ~52-kDa protein from rat brain that bound specifically to the M_4i3 loop. Sequencing identified the protein as eEF1A2, and this was confirmed by probing the M_4i3 loop affinity isolate with an eEF1A2 antibody. The M_4i3 loop and eEF1A2 recombinant proteins directly interacted *in vitro*; thus, the interaction does not require post-translational modifications. eEF1A2 did not interact with the M_2i3 loop or GST in any of these assays, demonstrating the specificity of this interaction. In the rat striatum, M_4 and eEF1A2 colocalized in the soma and neuropil, providing multiple sites for this interaction to occur in neurons. eEF1A2 co-immunoprecipitated with the endogenous M_4 mAChR, but not the M_1 mAChR, demonstrating a physical interaction under physiological conditions. Thus, these data identify eEF1A2 as a novel and specific binding partner for the M_4 receptor subtype.

To elucidate the potential functional importance of this novel interaction, we tested the hypothesis that the M_4i3 loop can act as a GEF for eEF1A2 based on ability of the M_4 mAChR to act as a GEF for $G_{i/o}$. Specifically, the portion of the M_4i3 loop used in our initial brain pull-down assays has been reported to be involved in the GEF activity of $G_{i/o}$ (31, 32). To test our hypothesis, we performed a nucleotide exchange assay using the fluorescent GDP analog, mantGDP, and recombinant eEF1A2. We established that recombinant eEF1A2 binds nucleotides and requires glycerol as been reported for the endogenous protein. Furthermore, we found that eEF1Bα increases nucleotide exchange of recombinant eEF1A2, which is consistent with previous reports on eEF1A (35, 36). The M_4i3 loop also significantly increases nucleotide exchange of recombinant eEF1A2. Thus, the M_4i3 loop is a GEF for eEF1A2. This raises the question why eEF1A2 would require another GEF *in vivo*.

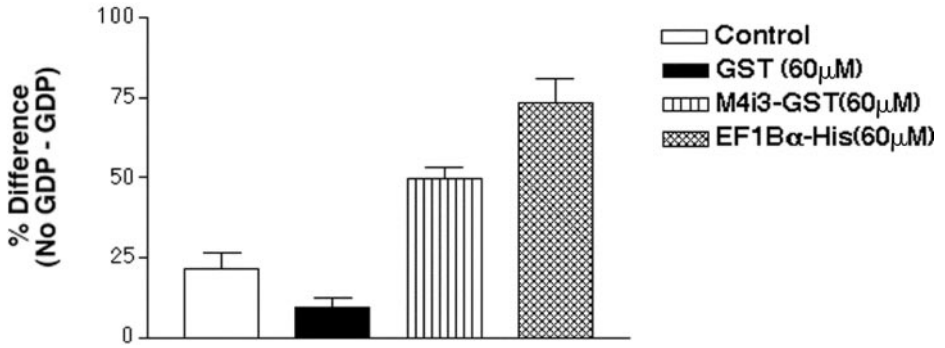

FIG. 6. **Regulation of eEF1A2 nucleotide exchange by the M_4i3 loop.** 10 μM eEF1A2 and 5 μM mantGDP were combined with 60 μM amount of eEF1Bα-His, M_4i3-GST, or GST. There were no significant differences in fluorescence between these conditions (data not shown). In parallel, excess unlabeled GDP was added to another set of identical solutions. We calculated the percentage difference of the fluorescence (*F*) between the solutions with and without unlabeled GDP. When unlabeled GDP was added to mantGDP-eEF1A2 (*control*), there was a ~20% decrease in fluorescence. In the presence of eEF1Bα-His, an ~80% decrease in fluorescence was observed. Similarly, the M_4i3-GST significantly decreased the fluorescence by 50%. These data reveal that eEF1Bα-His and the M_4i3-GST both accelerate the nucleotide exchange of eEF1A2. This graph represents four separate experiments in duplicate (*, $p < 0.005$). Fluorescence (*F*) = *F* [mantGDP + eEF1A2 (+ eEF1Bα, M_4, or GST)] − *F*[mantGDP (+ eEF1Bα, M_4, or GST)].

Although the expression of eEF1Bα in the brain is unknown, in human fibroblast cells, the concentration of eEF1A is ~5–10 fold higher than eEF1Bα, and there are subcellular regions that only express eEF1A (37). This suggests that other factors, such as the M_4 mAChR, may act to regulate nucleotide exchange of eEF1A2 in compartments lacking eEF1Bα.

eEF1A2 functions as an essential factor in protein synthesis as demonstrated by its activity in poly(U)-directed polyphenylalanine synthesis assay (38). Its proposed mechanism of action is as follows. eEF1A2-GTP binds amino acid-tRNA and transports it to the ribosome. The hydrolysis of GTP by eEF1A2 allows the correct amino acid to add to the nascent polypeptide chain, and eEF1A2-GDP is released from the tRNA. eEF1A2-GDP then exchanges GDP for GTP to repeat the cycle (34). Thus, if M_4 increases the nucleotide exchange of eEF1A2 *in vivo*, it would increase translation. It has been previously reported that muscarinic activation increases dendritic translation (39), but the mAChR subtypes necessary for this effect have not been identified. The colocalization of M_4 and eEF1A2 in the neuropil supports the hypothesis that the interaction between M_4 and eEF1A2 may be a mechanism for direct muscarinic modulation of dendritic translation. Furthermore, adrenergic receptors have been demonstrated to interact with the α subunit of eIF2B, suggesting that other GPCRs may directly regulate translation (40). Because agonist stimulation had no effect on the physical association between eEF1A2 and M_4 in our co-immunoprecipitation assay, it is still unclear what activates the eEF1A2 GEF activity of M_4. One possibility is that a conformational change upon agonist stimulation induces the eEF1A2 GEF activity of M_4, but has no effect on the physical association between these two proteins. For example, Lyn, an tyrosine kinase, physically associates with the α-amino-3-hyroxy-5-methyl-4-isoxazole propionate receptor, a ligand-gated cation channel, regardless of whether the receptor is stimulated. However, stimulation of the α-amino-3-hyroxy-5-methyl-4-isoxazole propionate receptor does activate the tyrosine kinase activity of Lyn, which activates downstream signaling pathways (41). Another possibility is that stimulation of multiple neurotransmitter systems are required to stimulate the eEF1A2 GEF activity of M_4. For example, Feig and Lipton (39) reported muscarinic stimulation of dendritic translation requires the co-stimulation of *N*-methyl-D-aspartate (NMDA) receptors.

Our data demonstrate a novel difference between M_4 and the closely related subtype M_2. M_4 and M_2 have high amino acid homology and both activate $G_{i/o}$. The lack of subtype specific agonists has impeded the elucidation of differences between M_2 and M_4 signaling pathways (42). However, the recent generation of M_2 and M_4 knockout mice does support the hypothesis M_2 and M_4 activate different signaling pathways. M_2 knockout mice have reduced muscarinic induced bradycardia, hypothermia, tremor, and analgesia, whereas M_4 knockout mice are similar to wild-type littermates in these assays (43, 44). Some of these behavior differences may be the result of differences in M_2 and M_4 expression in certain tissues. For example, previous studies have shown the heart expresses almost exclusively M_2 receptors (45), which explains the differences in muscarinic induced bradycardia between the M_2 and M_4 knockout mice. However, both M_2 and M_4 are expressed in the striatum, a region known to be critical in extrapyramidal motor activity, and both have been localized to cholinergic terminals in the striatum (29, 46). A recent report using these knockout mice has demonstrated that M_4, and not M_2, is an autoreceptor in the striatum (46). Behaviorally, the M_4 knockout mice showed abnormalities in locomotor activity. Specifically, the M_4 knockout mice have a significant increase in base-line locomotor activity and increased sensitivity to D1 agonist, a locomotor stimulant (47). Recently, a spontaneous mutation in mice resulting in the *wasted* phenotype was identified as a mutation in the eEF1A2 gene, which abolished eEF1A2 expression (48). Interestingly, the *wasted* mice have motor deficits and neurodegeneration. Thus, our data suggest eEF1A2 may play a role in the differential signaling of M_2 and M_4.

In conclusion, we demonstrated that the M_4 mAChR acts as a GEF for eEF1A2 via direct binding to the M_4i3 loop. This may provide a mechanism by which muscarinic activation can increase dendritic translation and may also allow for muscarinic regulation of other functions of eEF1A2. eEF1A has been demonstrated to bind and bundle actin (49), bind microtubules (50, 51), bind calmodulin (52), and regulate apoptosis (53). Also, this interaction may provide a molecular mechanism underlying functional differences between the two closely related mAChR subtypes, M_2 and M_4.

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