Muscarinic Receptor Stimulation Induces Translocation of an α-Synuclein Oligomer from Plasma Membrane to a Light Vesicle Fraction in Cytoplasm*

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The close correspondence between the distribution of brain α-synuclein and that of muscarinic M1 and M3 receptors suggests a role for this protein in cholinergic transmission. We thus examined the effect of muscarinic stimulation on α-synuclein in SH-SY5Y, a human dopaminergic cell line that expresses this protein. Under basal conditions, α-synuclein was detected in all subcellular compartments isolated as follows: plasma membrane, cytoplasm, nucleus, and two vesicle fractions. The lipid fractions contained only a 45-kDa α-synuclein oligomer, whereas the cytoplasmic and nuclear fractions contained both the oligomer and the monomer. This finding suggests α-synuclein exists physiologically as a lipid-bound oligomer and a soluble monomer. Muscarinic stimulation by carbachol reduced the α-synuclein oligomer in plasma membrane over a 30-min period, with a concomitant increase of both the oligomer and the monomer in the cytoplasmic fraction. The oligomer was associated with a light vesicle fraction in cytoplasm that contains uncoated endocytotic vesicles. The carbachol-induced alteration of α-synuclein was blocked by atropine. Translocation of the α-synuclein oligomer in response to carbachol stimulation corresponds closely with the time course of ligand-stimulated muscarinic receptor endocytosis. The data suggest that the muscarine receptor stimulated release of the α-synuclein oligomer from plasma membrane, and its subsequent association with the endocytotic vesicle fraction may have a role in muscarine receptor endocytosis. We propose that its function may be a transient release of membrane-bound phospholipase D2 from α-synuclein inhibition, thus allowing this lipase to participate in muscarinic receptor endocytosis.

The neuronal protein α-synuclein, recently implicated in the pathogenesis of Parkinson’s disease (1–3), has a brain distribution closely matching that of muscarinic M1 and M3 receptors and the muscarine receptor-linked enzymes phospholipase C and protein kinase C (4). The proximity of α-synuclein to brain muscarinic receptors and related enzymes suggests that this protein could contribute to cholinergic function. To evaluate this possibility, we investigated the effect of muscarinic receptor stimulation by the cholinergic agonist carbachol on the subcellular distribution of endogenous α-synuclein in the human neuroblastoma cell line SH-SY5Y. This dopaminergic cell line shares many properties with dopamine neurons of substantia nigra pars compacta, the major locus of neurodegeneration in Parkinson’s disease, including the expression of M1 and M3 receptors (5). We now report that carbachol induces a translocation of a 45-kDa α-synuclein oligomer from plasma membrane to a light vesicle fraction in cytoplasm, with a time course and subcellular localization matching that of muscarinic receptors during carbachol-stimulated endocytosis. Previously, synucleins have been demonstrated to be endogenous inhibitors of phospholipase D2 (PLD2) (6). Furthermore, activation of PLD isoforms, including PLD2, stimulates endocytosis (7–9). Thus, the present data are consistent with a model of muscarinic receptor endocytosis in which the release of α-synuclein from plasma membrane transiently disinhibits membrane-bound PLD2, freeing this lipase to function in ligand-stimulated muscarinic receptor endocytosis.

EXPERIMENTAL PROCEDURES

Materials—Anti-synuclein-1 (Syn-1) monoclonal antibody, annexin II monoclonal antibody, and goat anti-mouse IgG polyclonal horseradish peroxidase conjugate were from BD Transduction Laboratories; ECL detection reagents were from Amersham Pharmacia Biotech; 10× SDS/Tris glycine electrophoresis running buffer and non-fat dry milk were from Bio-Rad; 4–20% Tris glycine gels, XCell II Mini-Cell electrophoresis system and Blot Module, PVDF membrane/filter paper, MultiMark Multicolored and See-Blue standards were from NOVEX; 10× Tris glycine Transfer Buffer was from Quality Biological Inc.; phosphate-buffered saline (PBS) and Dulbecco’s modified Eagle’s medium were from Cellgro-Mediatech; fetal bovine serum was from Life Technologies, Inc.; and protease inhibitor mixture was from Roche Molecular Biochemicals.

Buffers—The following buffers were used: cell wash buffer (0.25 M sucrose, 10 mM HEPES, pH 7.5); sucrose homogenization buffer (0.25 M sucrose, 10 mM HEPES, 1 mM MgCl2, protease inhibitor mixture, pH 7.5); high density sucrose buffer (2.4 M sucrose, 10 mM HEPES, 1 mM MgCl2, protease inhibitor mixture, pH 7.5); freezing buffer (6 mM Tris-HCl, 4 mM EGTA, 1 mM MgCl2, 10% glycerol, pH 7.4); and 2× SDS sample buffer (126 mM Tris-HCl, 4% SDS, 20% glycerol, 0.0025% bromophenol blue, pH 6.8).

Cell Culture and Lysis—Human dopaminergic neuroblastoma SH-SY5Y cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum and kept at 37°C in humidified 5% CO2, 95% air. Cells grown on 100-mm plates to 80–90% confluence were rinsed with 1 ml of ice-cold wash buffer. Cells were fractionated according to a modification of the procedures of Fleischer and Kervina (10) as follows. Cells from each plate were fractionated according to a modification of the procedures of Fleischer and Kervina (10) as follows. Cells from each plate were

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1 The abbreviations used are: PLD, phospholipase D; PI, phosphatidylinositol; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline.

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scraped with 0.5 ml of ice-cold sucrose homogenization buffer into a 5-ml glass homogenization vessel and lysed by 10 strokes of a glass pestle with a Dounce ball tip. Homogenates from each plate were centrifuged at 14,500 × g for 10 min (3500 rpm, Beckman J-21, JA-20 rotor). The post-nuclear supernatants were transferred to other tubes and kept on ice cold for later separation of vesicles and cytoplasm. Nuclei and plasma membranes were isolated from the resulting pellets.

Isolation of Plasma Membrane and Nuclear Fractions—The pellet from the initial centrifugation was resuspended in 1 ml of ice-cold sucrose homogenization buffer. A volume of 1.62 ml of ice-cold high density sucrose buffer was added to the solution containing the resuspended pellet, producing a final concentration of 1.6 M sucrose. A two-step gradient was set up by layering a sufficient volume of 0.25 M sucrose buffer over the 1.6 M sucrose suspension to fill the tube and then centrifuged at 70,900 × g R, (24,000 rpm, SW 25.2) rotor for 70 min. The band at the gradient interface (0.25 and 1.6 M sucrose) was enriched in plasma membrane. The plasma membrane-containing band was carefully transferred to other tubes after removal by aspiration of the top layer of 0.25 M sucrose buffer. The pellet produced after the gradient centrifugation contained the nuclear fraction.

Isolation of Vesicle and Cytoplasmic Fractions—Two vesicle and cytoplasmic fractions were isolated by different centrifugation procedures used with matched samples of post-nuclear supernatant. A vesicle fraction termed V1 was isolated by ultracentrifugation of the post-nuclear supernatant at 100,000 × g for 1 h. The pellet of this ultracentrifugation contained vesicles in the density range of synaptic vesicles (11). The resulting pellet was resuspended in freezing buffer, which stabilizes the sample for freezing at −80 °C or could be analyzed immediately. The supernatant of this centrifugation is the cytoplasmic fraction we termed S1. Matched culture plates were used to sediment a larger pellet, producing a final concentration of 1.6 M sucrose. A density sucrose buffer was added to the solution containing the resuspended pellet, producing a final concentration of 1.6 M sucrose. A two-step gradient was set up by layering a sufficient volume of 0.25 M sucrose buffer over the 1.6 M sucrose suspension to fill the tube and then centrifuged at 70,900 × g R, (24,000 rpm, SW 25.2) rotor for 70 min. The band at the gradient interface (0.25 and 1.6 M sucrose) was enriched in plasma membrane. The plasma membrane-containing band was carefully transferred to other tubes after removal by aspiration of the top layer of 0.25 M sucrose buffer. The pellet produced after the gradient centrifugation contained the nuclear fraction.

RESULTS AND DISCUSSION

The α-synuclein monoclonal antibody consistently detected two molecular species of this protein in the SH-SY5Y whole cell lysate. The α-synuclein monomer migrated in gel electrophoresis at its expected position corresponding to an apparent molecular mass of ~19 kDa (14) and a second α-synuclein signal was detected at ~45 kDa. The 45-kDa band appears to be an oligomer containing at least one α-synuclein molecule, as it can be non-enzymatically cleaved under stringent reducing conditions, resulting in a quantitative increase in the monomer (15) (Fig. 1). The vigorous reducing conditions required for cleavage of this oligomer suggest that the bond between molecules may be an azo bond (16). This linkage may be similar to the dityrosine bonds recently characterized as being formed after tyrosine nitration (17).

The distribution of the two forms of α-synuclein in the various cellular compartments was determined by subcellular fractionation of whole cell lysate both under basal conditions and in response to carbachol stimulation for incubation intervals of up to 30 min. The post-nuclear supernatant was used to isolate two vesicle fractions V1 and V2 from matched sets of samples as described under “Experimental Procedures.” The α-synuclein in the vesicle fraction we termed V1 was shown in previous work to be associated exclusively with vesicles of densities 1.0995–1.250 g/ml and to distribute identically with synaptic vesicle-associated protein, SNAP-25 (11), thus providing strong evidence that all of the α-synuclein in this fraction is bound to synaptic-like vesicles. The V2 fraction contains, in addition to the vesicle of the V1 fraction, a lighter vesicle fraction that includes endocytotic vesicles that have lost their clathrin coating (12). The supernatants from each centrifugation condition were the cytoplasmic fractions termed S1 and S2, respectively.

The efficacy of the separation of the cellular fractions was demonstrated by the relative concentrations of annexin II, a
marker of plasma membrane and early endosomes (13). Annexin II was highly concentrated in plasma membrane compared with whole cell lysate and was weakly present in the cytosolic S1 fraction from unstimulated cells (Fig. 2). With a 4-fold increase in sample protein, however, the annexin II signal was clearly detected in the S1 fraction of unstimulated cells and increased with carbachol stimulation (Fig. 3). This increase suggests that the annexin II antibody detected the increase in early endosomes containing muscarinic receptors, which have been demonstrated to increase in response to carbachol stimulation (12). Annexin II was absent in the V1 fraction, consistent with its localization in lipids of early endosomes but not synaptic vesicles (Fig. 2). Thus, the detection of annexin II exclusively in the plasma membrane and the S1 cytoplasmic fractions validates the methods of subcellular fractionation used in this experiment.

All membrane fractions, i.e. the plasma membrane fraction and vesicle fractions, contained only the 45-kDa form of α-synuclein (Fig. 4, a and b). In contrast, the cytoplasmic and nuclear fractions often contained both the monomer and the heavier species (Fig. 4, c and d). The lack of the α-synuclein monomer in all of the membrane fractions suggests that the membrane-associated α-synuclein is exclusively oligomeric in SY5Y cells. The 45-kDa species was evident in the S1 supernatant but weak or absent in the S2 supernatant, indicating that a subcellular component containing this molecule is not sedimented by the conditions yielding the V1 fraction but can be sedimented by the higher gravitational force centrifugation that yields the V2 pellet (Fig. 5). Thus, by comparing the results of the different centrifugation conditions, we conclude that the soluble α-synuclein monomer is found in the aqueous cytosol, and the 45-kDa oligomer is associated with two distinct vesicle populations differentiated by density.

A previous investigation (18) identified an oligomer of purified recombinant α-synuclein with an apparent molecular mass of ~45 kDa, similar to the membrane-associated oligomer of the present study. The α-synuclein oligomer found in this study was formed only when α-synuclein was incubated with artificial vesicles containing phosphatidylinositol (PI). The α-synuclein monomer bound only to small unilamellar vesicles containing at least 20% acidic phospholipids, whereas the polymerization of vesicle-bound α-synuclein occurred only if the phospholipid was PI. These results suggest that the oligomeric α-synuclein detected in the SY5Y cellular environment is likely to be a homo-oligomer that binds solely to membranes enriched in phosphatidylinositols. The oligomer of purified α-synuclein detected in vitro was described as a homodimer (18); however, it seems more plausible that both the recombinant oligomer and the molecule presently detected in SY5Y cell lysate are homotrimers of α-synuclein. The molecular mass of endogenous α-synuclein monomer as determined by mass spectrometry is 14,681 (19), but it migrates in gel electrophoresis with an apparent mass of ~19 kDa, due to the highly acidic terminal sequence of this natively unfolded molecule (20). Upon vesicle binding, α-synuclein adopts a stable α-helical structure (18). This conformational change from an elongated molecule to one with considerable secondary structure can be predicted to be accompanied by a change to more conventional electrophoretic migration characteristics. A molecule of ~45 kDa would be more accurately 3 times the molecular weight of the α-synuclein monomer, thus being more consistent with a homotrimer. Alternatively, the higher molecular weight species
may be a heteromer of α-synuclein bound to an unidentified binding partner.

Nuclear binding of α-synuclein has been controversial (21, 22). We detected both the α-synuclein monomer and the 45-kDa species in the nuclear fraction (Fig. 4d). In addition to subcellular fractionation, we also visualized the distribution of α-synuclein by immunocytochemistry (Fig. 6). By this method also, α-synuclein was detected in the nucleus and cytoplasm of the SY5Y cells. Typically, the Western blot nuclear signal from the 45-kDa species was much stronger than that of the monomer. In the original characterization of α-synuclein in vivo, Maratoeax et al. (21) reported α-synuclein antigenicity over discrete portions of the nuclear envelope of neurons of the Torpedo electric organ, with the antigen signal diminishing over a 10-μm gradient toward the interior of the nucleus. The present findings are consistent with the 45-kDa α-synuclein oligomer being associated with the nuclear membrane and the soluble monomer existing in the nucleoplasm.

Carbachol stimulation induced a consistent decrease in the 45-kDa signal detected in the plasma membrane fraction (Fig. 7a) and a concomitant increase in both the oligomer and the monomer in the cytoplasmic fraction S1 (Fig. 7b). There was little or no alteration in the amount of α-synuclein in the vesicle V1 fraction in response to carbachol stimulation (Fig. 7c), although there was abundant α-synuclein in this fraction, which includes the synaptic-like vesicles. In contrast to the V1 fraction, a clear and marked increase in the α-synuclein signal was detected in the V2 vesicle fraction (Fig. 8). A comparison of results from the two centrifugation conditions suggests that carbachol stimulation triggers a translocation of the α-synuclein oligomer from the plasma membrane to a light vesicle fraction in cytoplasm that is sedimented in the V2 but not the V1 fraction. Typically, however, the changes in the α-synuclein oligomer associated with the light vesicle fraction were more evident when this lipid fraction remained in the S1 cytoplasm (Fig. 9). The overall protein concentrations were measured and found to beunal-

**FIG. 5.** Comparison of the supernatant fractions S1 and S2 under basal conditions. Typically the 45-kDa α-synuclein oligomer that is present in the S1 fraction (a) is weak or absent in the S2 fraction (b), indicating a low density particulate component that remains in the S1 fraction is sedimented from the S2 into the V2 by the longer centrifugation duration under higher gravitation forces.

**FIG. 6.** Expression of α-synuclein in SY5Y cells. Cultured SY5Y cells were fixed in 4% paraformaldehyde (in 0.1 M PBS, pH 7.4) for 10 min at 4 °C, followed by washes in PBS. Slides were incubated 12 h at 4 °C in 0.01 M PBS containing 1:800 dilution of the Syn-1 monoclonal antibody (Vector Laboratories). α-Synuclein immunoreactivity was clearly evident in nuclei and cytoplasm of these cells. Black arrows, examples of α-synuclein-stained cell nuclei; white arrows, examples of α-synuclein-stained cytoplasm and processes.

**FIG. 7.** Effect of carbachol on α-synuclein distribution in cellular compartments. Representative examples of plasma membrane (a), cytoplasmic (S1) fraction (b), and vesicle (V1) fraction (c). There was a consistent decrease in the 45-kDa α-synuclein oligomer in plasma membrane over the 30 min of carbachol stimulation. Concomitant with the decrease in the α-synuclein oligomer in plasma membrane, there was also a consistent increase in this species in the S1 cytoplasmic fraction. The α-synuclein monomer also increased over the same period in the cytoplasmic fraction. Although there is abundant oligomeric α-synuclein associated with the synaptic-like vesicles in V1, the response to carbachol stimulation ranged from no effect to a slight increase.
Carbachol Stimulates α-Synuclein Translocation

Muscarinic receptors in many cell types, including SY5Y cells, undergo desensitization induced by carbachol stimulation that is mediated by ligand-stimulated, clathrin-dependent endocytosis (12, 23). Endocytic vesicles containing muscarinic receptors translocate from the plasma membrane to the cytoplasm and rapidly lose their clathrin coats (23–25). The uncoated endocytic vesicles are contained in the light membrane fraction that is included in the V_2 pellet (12). We determined also that the α-synuclein signal increase in the cytoplasmic S_1 fraction was matched by an increase in the annexin II signal, a marker for early endosomes (13) (Fig. 3).

The carbachol-induced α-synuclein translocation from plasma membrane to the light vesicle compartment matches the time course and subcellular translocation of muscarinic receptors during ligand-stimulated muscarinic receptor endocytosis (12). The internalization of α-synuclein was blocked completely by atropine (Fig. 10), indicating the specificity of this response to muscarinic receptor stimulation. Collectively, these results provide strong evidence that α-synuclein participates in ligand-stimulated muscarinic receptor endocytosis.

A role for α-synuclein in muscarinic receptor endocytosis is consistent with its co-localization with muscarinic receptors and related enzymes in brain and fits well with the first reported physiological function of synucleins as potent endogenous inhibitors of phospholipase D_2 (PLD_2) (6). Muscarinic stimulation is well documented to activate PLD in various cell types, including SY5Y cells, although distinctions have not been characterized between the PLD_1 and PLD_2 isoforms (26, 27). PLD_2 is a constitutively active isoform of PLD that is localized primarily along the plasma membrane (9). The potent inhibition of PLD_2 by synucleins in vitro (6) suggests that its basal activity in vivo may be a result of tonic inhibition by synucleins. Overexpression of PLD_2 in cell culture induces endocytosis coincident with a redistribution of PLD_2 from plasma membrane to submembrane vesicles and simultaneously induces actin polymerization, which is characteristic of the endocytotic phase of synaptic transmission (9). Transient disinhibition of post-synaptic PLD_2 after cholinergic stimulation would accomplish several requirements of clathrin-mediated receptor endocytosis. Phospholipase D has been implicated in several steps of endocytosis, including recruitment of coat assembly proteins (28, 29). The observation that the recruitment of adaptin protein-2 to plasma membrane prior to endocytosis does not require the GTPase ADP-ribosylation factor, unlike the PLD_1-controlled recruitment of adaptin protein-1 to the Golgi network, led to the speculation that adaptin protein-2 attachment to plasma membrane may be under the control of a “constitutively active phospholipase D” (30), despite the fact that one had not been discovered at the time.

Membrane-bound disinhibited PLD_2 is also well positioned to hydrolyze plasma membrane phosphatidylcholine (PC) at the endocytotic vesicle point of attachment to plasma membrane, thus releasing the vesicle into cytosol after it is closed at its base by the GTPase dynamin (31). Dynamin does not pinch off the vesicle, and at present, the mechanism for vesicle detachment is not known. Membrane lysis by PLD_2-mediated PC hydrolysis could provide an additional advantage of liberating free choline, approximately half of which is lost during cholinergic neurotransmission. Cholinergic stimulation of membrane

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**FIG. 8.** Effect of carbachol stimulation on the cytoplasmic S_2 and vesicle V_2 compartments. a, as shown in 7b also, both oligomers and monomers increase in the S_1 fraction in response to carbachol stimulation. c, in contrast, the α-synuclein oligomer signal from the cytoplasmic S_2 fraction is almost undetectable in the control lane (C) and increases only slightly with carbachol stimulation (probably reflecting the presence of unsedimented light vesicles). The monomer, however, increases as it does in the S_1 fraction. The α-synuclein oligomer in the V_2 fraction (d), in contrast with that in the V_1 fraction (b), shows a clear increase in response to carbachol stimulation. The V_2 fraction includes the light vesicle fraction that remains in the cytoplasmic S_1 fraction.

**FIG. 9.** Carbachol-induced translocation of α-synuclein from plasma membrane to a light vesicle fraction in cytoplasm. The translocation of the α-synuclein oligomer from plasma membrane (a) to the light vesicle fraction was typically more distinct when this fraction remained in the supernatant S_1 (b), where its appearance emerged in contrast to a typically low basal signal, than when it was sedimented into the V_2 fraction (compare with Fig. 8d) and the carbachol-induced increase appeared against a higher basal signal of the α-synuclein oligomer. The vesicle fraction V_1 (c) remains unaltered.
induced changes in α-synuclein oligomer and monomer in cytoplasm (a) and decrease in α-synuclein oligomer in plasma membrane (b).

PC hydrolysis by PLD has been proposed by several groups (27, 32, 33) as a source of free choline for acetylcholine synthesis. The proposed modulation of cholinergic receptor endocytosis by the transient release of PLD₂ from α-synuclein inhibition suggests a compact and parsimonious sequence of linked events for processes occurring during cholinergic neurotransmission.

The present data do not resolve the issue of whether the α-synuclein oligomer remains associated with membrane throughout the entire process of endocytotic vesicle formation or is released from plasma membrane before reattaching to the endocytotic vesicle. Several lines of evidence from our laboratory and others favor a model in which muscarinic stimulation induces the release of α-synuclein from plasma membrane, with a concomitant conversion of the oligomer to soluble monomers prior to a reattachment and oligomerization of α-synuclein at the endocytotic vesicle. We observed a carbachol-induced increase in the soluble monomer in the cytoplasmic fraction that is consistent with a muscarinic receptor-stimulated cleavage of lipid-bound α-synuclein oligomer and release of the soluble monomer into cytosol (Figs. 7–9). The recent findings that G-protein receptor kinases phosphorylate synucleins and reduce their binding affinity for phospholipid (34) also support the hypothesis that α-synuclein is first released from plasma membrane before reattaching to the endocytotic vesicle membrane in response to muscarinic stimulation. Furthermore, Marrateaux and Scheller (4) noted that a 45-kDa form of α-synuclein was converted to the 19-kDa form by stimulation of phospholipase C, the lipase coupled to muscarinic receptors. Taken together these data suggest that the carbachol-stimulated increase in the cytoplasmic monomer may represent a transition state for α-synuclein in its translocation from plasma membrane to uncoated endocytotic vesicles.

Carbachol stimulation induced changes in α-synuclein in the nucleus, as well as in the cytoplasmic and membrane fractions. The most consistent change was a decrease in the α-synuclein monomer over the 30 min of stimulation (Fig. 11a). It is unclear whether this decrease contributes to the increase in the cytoplasmic monomer. Although there were no consistent changes in the 45-kDa species, two experiments showed a biphasic effect of carbachol stimulation, with a transient decrease at 10 min and a return to a level greater than the basal value by 30 min. This effect was especially clear in this experiment in which we also detected a strong signal between 30 and 36 kDa after 10 min of carbachol exposure. C, control lane.

In conclusion, we have determined that endogenous α-synuclein in the human SY5Y cell exists in at least two normal states, a soluble monomer and an oligomer of ~45 kDa that is exclusively associated with lipid membranes. This distinctive subcellular distribution of α-synuclein suggests that it is the oligomer that mediates all membrane-linked functions of α-synuclein. The present data support a model in which the α-synuclein oligomer functions in vivo to inhibit PLD₂ at the plasma membrane, consistent with the synuclein inhibition of this PLD isoform in vitro. In response to carbachol stimulation, the α-synuclein oligomer translocates from plasma membrane to a light vesicle fraction in cytoplasm with a time course and subcellular localization corresponding to that of muscarinic receptors during ligand-stimulated endocytosis. The data suggest that muscarinic stimulation triggers the release of the α-synuclein oligomer from plasma membrane and transiently inhibits PLD₂, freeing this lipase to mediate several processes of muscarinic receptor endocytosis.

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