Fluorescence Studies Suggest a Role for α-Synuclein in the Phosphatidylinositol Lipid Signaling Pathway†

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ABSTRACT: α-Synuclein plays a key role in the pathogenesis of many neurodegenerative diseases. To date, its cellular role has yet to be determined, although it has been proposed to be connected to calcium and G protein-mediated dopamine signaling. α-Synuclein is known to bind strongly to model membrane surfaces where it may interact with other membrane-associated proteins. Here, we find that the membrane association of α-synuclein is enhanced by the presence of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] and Ca^{2+}. We also find that α-synuclein interacts with high affinity with the G protein-regulated enzyme phospholipase Cβ_2 (PLCβ_2), which catalyzes the hydrolysis of PI(4,5)P_2. Binding of α-synuclein to PLCβ_2 reduces its catalytic activity by 50%, but causes its level of activation by G$\beta\gamma$ subunits to increase from 4- to 24-fold. This effect is greatly reduced for A53T α-synuclein, which is a mutant associated with familial Parkinson's disease. PI(4,5)P_2 hydrolysis by PLCβ_2 results in an increase in the intracellular Ca^{2+} concentration, and we find that in cultured cells the presence of α-synuclein results in a 6-fold enhancement in the release of Ca^{2+} from intracellular stores in response to agents that release G$\beta\gamma$ subunits relative to controls. α-Synuclein also enhances the increase in the level of inositol phosphates seen upon G protein stimulation, suggesting that it also may interact with PLCβ_2 in cells. Given that Ca^{2+} and dopamine regulation are mediated through PLCβ and G protein signals, our results suggest that α-synuclein may play a role in inositol phospholipid signaling.

α-Synuclein is the major component of proteinaceous inclusions characteristic of several neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, multiple-system atrophy, and dementia with Lewy bodies (see refs *1*−*3*). The level of expression of α-synuclein is highest in the brain, and it is also abundant in hematopoietic cells and platelets (see refs *4*−*6*). α-Synuclein is one of three family members of highly conserved vertebrate proteins (*5*−*8*). The high level of species conservation suggests that α-synuclein plays a specific cellular role, but to date, the function of this protein is unknown. Most evidence points toward a role in neuronal plasticity and synaptic function. Two point mutants of α-synuclein have been linked to familial Parkinson's disease (*9*, *10*), although the mechanism through which these small mutations can result in a disease state is unclear. α-Synuclein appears to be connected with dopamine signaling (*11*, *12*). Changes in α-synuclein expression have been found in early development and neurite growth (*5*, *7*, *13*). *In vitro* studies have shown that α-synuclein binds to and inhibits protein kinase C (PKC) (*14*) as well as phospholipase D2 (*15*), suggesting a role in signal transduction and vesicle trafficking.

α-Synuclein is purified as an unstructured protein, and on the basis of a weak homology to 14-3-3 proteins, it has been proposed that α-synuclein can serve as a chaperone or scaffolding protein (*14*). Purified α-synuclein aggregates over time (*16*, *17*), and this propensity to aggregate may underlie the formation of these neuropathogenic aggregates. Also, α-synuclein binds strongly to lipid membranes, and membrane binding will promote aggregation to a structured oligomer at higher but not lower protein concentrations (*11*, *18*−*21*).

Our laboratory has previously characterized the binding of α-synuclein to model membranes under nonaggregating conditions (*20*). Since α-synuclein may be linked to dopamine and Ca^{2+} signaling, we extended our binding studies to include the influence of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2].[1] PI(4,5)P_2 is a minor lipid component that can be hydrolyzed to yield Ins(1,4,5)P_3 which diffuses to receptors on the endoplasmic reticulum, causing the release of Ca^{2+} from intracellular stores.

The hydrolysis of PI(4,5)P_2 is catalyzed by phospholipase C enzymes. There are several types of mammalian PLCs with different mechanisms of regulation. The phospholipases of the PLCβ family are the key effectors of the Gα_q family of heterotrimeric G proteins, and the PLCϵ family can be stimulated by G proteins as well (for reviews, see refs *22* and *23*). It is notable that PLCβ enzymes are linked to

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[1] Abbreviations: PLCβ, mammalian phosphoinositide specific phospholipase C type β; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; InsP, inositol phosphate; LUVs, large unilamellar vesicles; coumarin, 7-(dimethylamino)coumarin-4-acetic acid succinimidyl ester; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine.

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dopamine receptors through G proteins (*24*), and as mentioned, α-synuclein also appears to be linked to dopamine signaling both in culture cells (*25*) and in knockout mice (*26*). It is also notable that ongoing research into the causes of Alzheimer's disease suggests a link between a disruption in Ca^{2+} homeostasis and disease onset (*27*, *28*). These studies led us to investigate whether α-synuclein could play a role in phosphatidylinositol signaling that is coupled to the dopamine−G protein−PLCβ pathway, ultimately leading to changes in Ca^{2+} signaling.

EXPERIMENTAL PROCEDURES

Expression and Purification of Proteins. Recombinant PLCβ enzymes, Gα_q, and G$\beta_1\gamma_2$ (here termed G$\beta\gamma$) were expressed in Sf9 cells using a baculovirus system (see refs *29* and *30*). Human α-synuclein and the A53T mutant were prepared by bacterial expression and purified as previously described (*15*, *20*). Protein identification and purity were confirmed by SDS−PAGE followed by Western blot analysis using a monoclonal antibody, LB409 (Zymed).

Membrane Binding Studies. Measurements of the level of binding of α-synuclein to freshly extruded large, unilamellar vesicles have been described previously (*20*). Briefly, changes in the emission energy emanating from the four tyrosine residues of α-synuclein are followed as lipid is added. The spectra are then corrected using control samples where buffer is substituted for protein. Binding isotherms were calculated by fitting the normalized change in emission energy as a function of lipid concentration (see ref *20*).

Subcloning of α*-Synuclein.* cDNA corresponding to human α-synuclein was subcloned into the Pet3a bacterial expression vector between the NdeI and HindIII sites and confirmed by DNA sequence analysis and by enyzmatic digestion. *Escherichia coli* BL21(DE3) cells were transformed with a positive clone containing the α-synuclein expression vector, Pet3a-asyn.

α-Synuclein was subcloned into the mammalian expression vector (pRC-CMV) by excising the α-synuclein insert from Pet3a-asyn between the XbaI and HindIII restriction enzyme sites and ligating it into a pRC-CMV vector (Invitrogen) which was previously digested with HindIII. Positive clones were screened initially by restriction enzyme digestion for length and orientation and then by DNA sequence analysis.

Cell Culture and Transfections. A10 cells from ATCC were maintained at 37 °C in 5% CO_2 in DMEM (Gibco BRL) replenished every 3−4 days and supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and penicillin/streptomycin. For the assessment of calcium release, PBS was supplemented with 0.1 g/L calcium chloride and 0.1 g/L magnesium sulfate. Transfections were carried out using Lipofectamine (Invitrogen) at 75−80% confluency, and expression was detected by immunofluorescence. PC12 cells were also cultured in DMEM supplemented with 10% equine serum, 5% FBS,100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were transfected with 10 μg of α-synuclein or pRC-CMV empty vector with Lipofectamine 2000.

Measurements of the Rate of Ca^{2+} *Release.* A10 cells were labeled with acetomethoxy (AM) ester of Calcium Crimson (Molecular Probes, Eugene, OR) by adding a small aliquot of a concentrated DMSO stock to a final concentration of 3 μM. Labeling was carried out on adherent cells prior to trypsinization, or cells were first lifted by trypsinization and then labeled. Cells were washed twice with PBS, incubated at 25 °C for 40 min in the dark with a fluorescent probe, and then washed twice with PBS. Cells were allowed to recover for 20 min before measurements were taken.

The Ca^{2+} response of 20 or 30 μL of fluorescently labeled A10 cells, diluted into 1 mL of PBS containing Ca^{2+} and Mg^{2+}, was measured within 2 min of the addition of increasing concentrations of carbachol or acetylcholine at 5 min intervals. The change in the integrated intensity, exciting at 575 nm and scanning from 600 to 700 nm, was monitored on a photon counting fluorometer (ISS, Inc., Urbana, IL). Changes in Ca^{2+} release of α-synuclein-transfected or control empty vector-transfected cells at a specific agonist concentration were calculated as a percentage of the initial fluorescence emission intensity. The ratio of the responses of α-synuclein-transfected cells to empty vector is presented.

Measurements of [^{3}*H]Inositol Phosphate (InsP) Production in Cells.* Cells were prelabeled with myo[^{3}H]inositol (1 μCi/mL) for 2 days in inositol-free medium, and incubated for 10 min at 37 °C with HBSS/LiCl before being challenged with agonists. Cells were incubated with an agonist (1 μM acetylcholine, 1 μM carbachol, 1 μM insulin, or 100 ng/mL epidermal growth factor) in the presence of LiCl for 30 min at 37 °C. In some studies, cells were incubated with 100 ng/mL pertussis toxin (PTX) for the last 16 h of labeling. Reactions were stopped by removing the incubation medium and lysing the cells in 1 mL of ice-cold methanol. After addition of 1 mL of chloroform and 0.5 mL of H_2O, phase separation was performed by centrifugation at 2000*g* for 10 min at 4 °C. The aqueous upper phase was applied to AG 1-X anion exchange columns to isolate myo[^{3}H]inositol phosphates (*31*).

PLCβ*−G*$\beta\gamma$ *Protein Activation Assays. In vitro* PLCβ activation assays were carried out as previously described (*29*) using varying amounts of purified protein as indicated and 60 μM sonicated lipid vesicles composed of POPS, POPE, and PI(4,5)P_2 [doped with [^{3}H]PI(4,5)P_2] at a molar ratio of 65:33:2.

Fluorescence Assays. The binding of α-synuclein to PLCβ_2 and to G$\beta\gamma$ subunits was assessed by labeling the latter two proteins with coumarin methoxy succinimidyl ester (Molecular Probes, Inc.) at a probe:protein molar ratio of 1:1 (see ref *29*). We found that addition of α-synuclein to the coumarin-labeled proteins resulted in a shift in emission energy and an increase in emission intensity that was not observed when buffer or a nonbinding partner was substituted for α-synuclein, and these shifts were used to construct binding curves. We note that binding of α-synuclein to G$\beta\gamma$ subunits was carried out using G$\beta_1\gamma_2$ prebound to lipid bilayers composed of 33% POPS (1,2-palmitoyloleoylphosphatidylserine) and 67% POPC (1,2-palmitoyloleoylphosphatidylcholine) to ensure solubility of the G proteins.

Secondary immunofluorescence was carried out by fixing with 4% formaldehyde, washing, permeabilizing with 0.1% Triton X-100, and blotting overnight with 5% goat serum, 1% BSA, and 50 mM glycine overnight before addition of the primary antibody [monoclonal anti-α-synuclein (LB409, Zymed)] and polyclonal anti-PLCβ_2 (Santa Cruz), followed by washing and then the addition of secondary antibodies

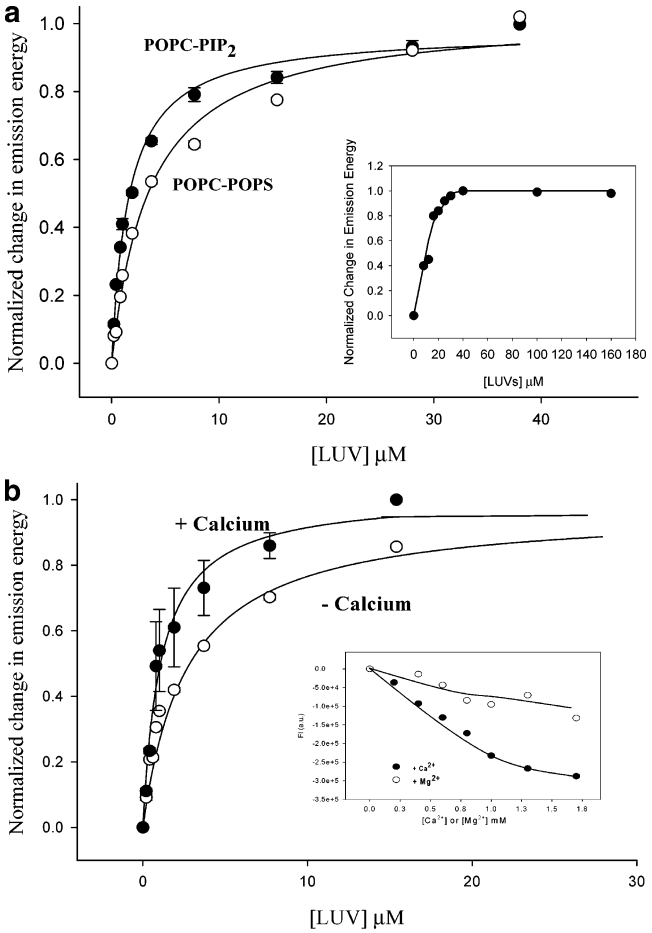

FIGURE 1: (a) α-Synuclein prefers membranes containing PI(4,5)P_2. Membrane binding of α-synuclein to large unilamellar vesicles composed of POPC and PI(4,5)P_2 (98:2) or POPC and POPS (98:2) in calcium-free buffer, where $n = 6$. The *Y*-axis refers to the shift in the center of spectral mass of α-synuclein as monitored by its intrinsic fluorescence (see Experimental Procedures). The standard error is shown. The inset shows the extended membrane binding curve. (b) A similar study as in panel a comparing the membrane binding of α-synuclein to POPC and PI(4,5)P_2 in Ca^{2+}-free buffer [20 mM Hepes, 150 mM KCl, and 1 mM EGTA (pH 7.2)] and a high-Ca^{2+} buffer [20 mM Hepes, 150 mM KCl, 1 mM EGTA, and 1.5 mM $CaCl_2$ (pH 7.2)]. The inset shows changes in the intrinsic fluorescence intensity of α-synuclein with added free Ca^{2+} or Mg^{2+}, where the free ion concentration was varied using 1 mM EGTA or EDTA, respectively.

(FITC anti-mouse and DsRed anti-rabbit). Cells were imaged on a Zeiss Axiovert 200M computational confocal microscope.

RESULTS

α-Synuclein Prefers To Bind to PI(4,5)P_2-Containing Membranes. We have previously found that α-synuclein binds strongly and with similar affinities to electrically neutral or anionic lipid membranes (*20*). We extended these binding studies to determine whether α-synuclein would prefer phosphatidylinositol lipids. To this end, we measured the membrane partition coefficient of purified α-synuclein for binding to large unilamellar vesicles (LUVs) composed of POPC and PI(4,5)P_2 (98:2) or POPC and POPS (98:2) by monitoring the shift in the intrinsic fluorescence to a higher emission energy as the protein becomes membrane-bound (see ref *20*). As shown in Figure 1a, we find that α-synuclein exhibits an ~2-fold preference for the membranes containing 2% PI(4,5)P_2; i.e., $K_p = 1.6 \pm 0.2$ μM for 2% PI(4,5)P_2, and $K_p = 3.6 \pm 0.4$ μM for 2% POPS.

Although α-synuclein does not contain any known Ca^{2+}-binding motifs, it does contain a highly anionic C-terminal region, and it has been shown that Ca^{2+} binds weakly to this region, promoting oligomerization (*32*). We monitored the intrinsic fluorescence of α-synuclein as a function of free Ca^{2+} concentration. We find changes in emission with an increase in free Ca^{2+} concentration that appeared to be complete by 2 mM Ca^{2+} (Figure 1b, inset). These spectral changes are also seen using free Mg^{2+} but to a lesser extent (Figure 1b, inset). On the basis of these results, we determined the sensitivity of α-synuclein membrane binding to the ionic environment by comparing its membrane partition to POPC and PI(4,5)P_2 (98:2) in buffer containing 0 and 500 μM free Ca^{2+}, where Ca^{2+}-bound α-synuclein would contribute to the binding. We find that the presence of Ca^{2+} increased the affinity (K_p) to 0.65 ± 0.17 μM. Thus, the combination of PI(4,5)P_2 and a high Ca^{2+} concentration increases the membrane partition coefficient ~5−6-fold.

α-Synuclein Enhances Activation of PLCβ$_2$ by Gβγ Subunits. The results in Figure 1 suggest that α-synuclein prefers membranes containing PI(4,5)P_2, so we then determined whether α-synuclein could affect the activities of PLCβ enzymes which hydrolyze PI(4,5)P_2 and are tied to dopamine and Ca^{2+} signaling (see the introductory section). There are four known mammalian PLCβ isoforms, PLCβ$_{1-4}$, which differ in their tissue distribution and their ability to be activated by Gα$_q$ and Gβγ subunits (see refs *22* and *23*).

We first added 2−50 nM α-synuclein to 4 nM PLCβ$_1$, which is strongly expressed in neuronal tissue. No changes in activity were observed. Similarly, no changes were observed with the broadly expressed PLCβ$_3$ (Figure 2a, left panel). However, nanomolar concentrations of α-synuclein resulted in a 50% inhibition of PLCβ$_2$ activity (Figure 2a, right panel), indicating that α-synuclein may be specifically altering the basal activity of this enzyme.

Since PLCβ$_2$ is activated by both Gα$_q$ and Gβγ subunits, we determined whether α-synuclein would affect its G protein activation. The addition of α-synuclein to PLCβ$_2$ did not affect the activation of these proteins by Gα$_q$ subunits (Figure 2b). In sharp contrast, the addition of α-synuclein substantially increased the level of activation of PLCβ$_2$ by Gβγ subunits. Under our assay conditions, PLCβ$_2$ shows a ~4-fold activation by Gβγ subunits (see refs *29*, *33*, and *34*). We found that adding increasing amounts of α-synuclein to the activated PLCβ$_2$−Gβγ complex results in a further increase in the level of PLCβ$_2$ activation, which reaches a maximum at 50 nM α-synuclein (Figure 2b). In an analogous series of studies, we inhibited PLCβ$_2$ by 50% by adding 50 nM α-synuclein, and then added increasing amounts of Gβγ subunits. This procedure resulted in a total 24-fold increase in the PLCβ$_2$ enzymatic activity (data not shown). We note that α-synuclein only affected the extent of Gβγ activation of PLCβ$_2$ rather than the concentration dependence of Gβγ activation.

Previous studies have identified a mutant of α-synuclein that underlies familial Parkinson's disease, A53T (*9*). To determine whether this mutant also affects the activity of PLCβ$_2$ or its ability to be activated by Gβγ, we repeated the studies depicted in Figure 2 using this mutant. We found that it too inhibited the basal activity of PLCβ$_2$ to a similar

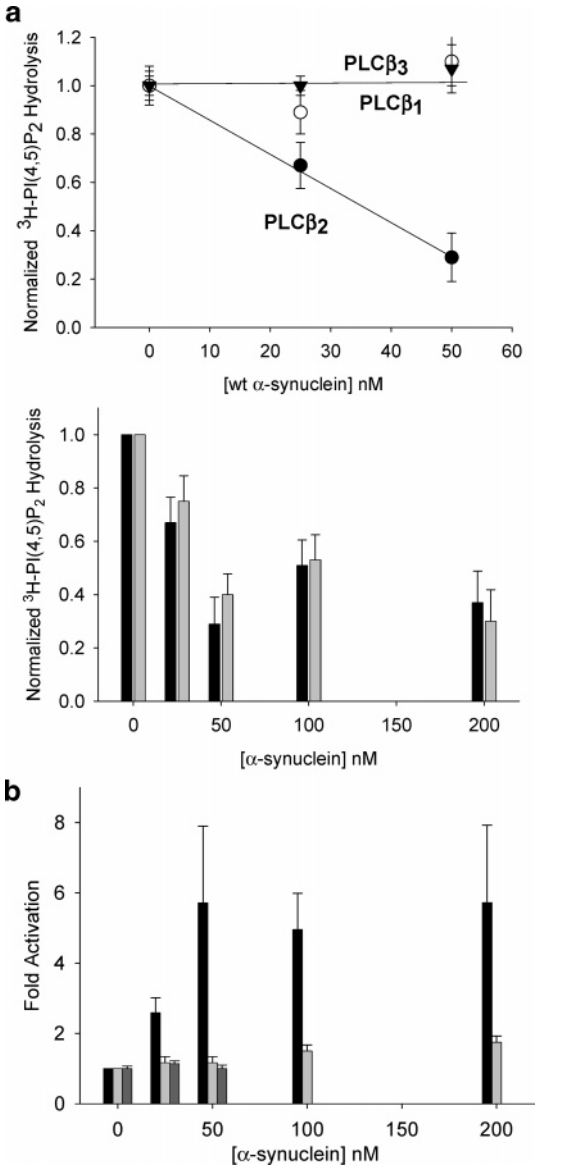

FIGURE 2: Wild-type and A53T α-synuclein affect the basal and G$\beta\gamma$-stimulated activity of PLCβ_2. (a) The upper panel compares the effect of wild-type α-synuclein on 4 nM PLCβ_{1-3} where initial activities in the absence of α-synuclein at 1 min gave between 0.5 and 2% hydrolysis of the total [^{3}H]PI(4,5)P$_2$. Comparative values for [^{3}H]Ins(1,4,5)P$_3$ for PLCβ_{1-3} are 189 ± 8, 157 ± 12, and 83 ± 5 cpm/mg, respectively. The lower panel shows the decrease in enzymatic activity of 4 nM PLCβ_2 with increasing amounts of α-synuclein (black bars) and A53T α-synuclein (gray bars), where the data are normalized to 1.0 at 0 nM α-synuclein. Each point is an average of three to six individual trials, and the standard error is shown. (b) Comparison of the effect of α-synuclein (black bars) and A53T α-synuclein (light gray bars) on the 100 nM G$\beta\gamma$−2 nM PLCβ_2 activated complex, where the extent of [^{3}H]Ins(1,4,5)-P$_3$ formation for all samples in the absence of α-synuclein was in the range of 40−50 cpm/mg. This concentration of G$\beta\gamma$ increased the level of [^{3}H]Ins(1,4,5)P$_3$ formation from 24 ± 4 to 102 ± 8 cpm/mg for a 45 s assay. Also shown is the effect of α-synuclein on 2 nM PLCβ_2 activated with 20 nM Gα_q subunits under the same conditions (dark gray bars).

extent (Figure 2a) and increased the level of G$\beta\gamma$ activation, but the magnitude of this increase was far smaller than that of wild-type α-synuclein (Figure 2b).

α-*Synuclein Binds Strongly to PLC*β_2. The enzymatic studies described above point to a strong physical interaction between α-synuclein and PLCβ_2. To determine whether this

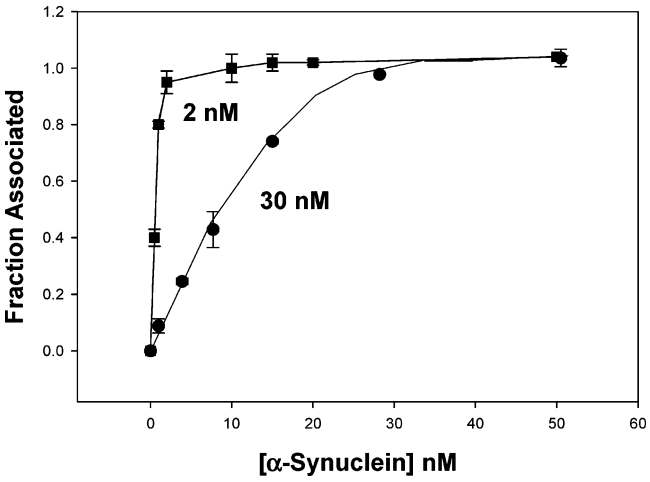

FIGURE 3: Fluorescence measurements of α-synuclein−PLCβ_2 association. Change in the emission intensity of (■) 2 and (●) 30 nM coumarin−PLCβ_2 upon the addition of purified α-synuclein, where the fraction association was calculated by the 18% increase in coumarin emission intensity relative to control samples that substituted buffer for α-synuclein (n = 3).

is the case, we attempted to measure the affinity between these proteins by measuring the change in fluorescence of coumarin-labeled PLCβ_2 as α-synuclein is incrementally added (see ref *35*). We found that the apparent K_d was not constant but shifted with the initial concentrations of coumarin-bound PLCβ_2 even with the smallest amount that would give reliable data (i.e., 2 nM PLCβ_2) (Figure 3). This result shows that the affinity between α-synuclein and PLCβ_2 is too strong to be measured by this technique (i.e., K_d < 1 nM), and we are viewing only stoichiometric binding. This strong binding may be related to the chaperone/scaffolding ability of α-synuclein and implies that in cells α-synuclein could form a complex with PLCβ_2 depending on localization and competing factors. Using this same methodology, we found that the dissociation constant of α-synuclein and G$\beta\gamma$ was measurable and we obtained a K_d of 34 ± 2 nM, which is much weaker than those for α-synuclein−PLCβ_2 complexes.

α-*Synuclein Accelerates the Release of Ca*$^{2+}$ *in Cultured Cells.* The studies described above suggest that α-synuclein may affect the regulation of PI(4,5)P$_2$ hydrolysis by PLCβ_2 and its activation by G$\beta\gamma$ subunits. If these interactions occurred in cells, then we would expect an increase in the rate of release of inositol phosphates produced by PLCβ_2 and subsequent intracellular Ca^{2+} upon stimulation when α-synuclein is present. Increased Ca^{2+} levels due to α-synuclein were tested by stimulating cultured cells that do not express α-synuclein with an agonist that will release G$\beta\gamma$ subunits, and comparing the release of intracellular Ca^{2+} to that in cells that have been transfected with α-synuclein. For these studies, we used rat aortic smooth muscle cells (A10) which contain muscarinic receptors (*36*) and express high levels of PLCβ_2, low levels of PLCβ_3, and no PLCβ_1 as determined by Western blot analysis (*37*). We could not detect α-synuclein in A10 cell lysates by Western blot analysis (data not shown). Our transfection procedure (see Experimental Procedures) gave an efficiency that ranged from 50 to 80% throughout the study as estimated by secondary immunofluorescence. In accord with previous reports, in transfected cells, α-synuclein was widely dispersed

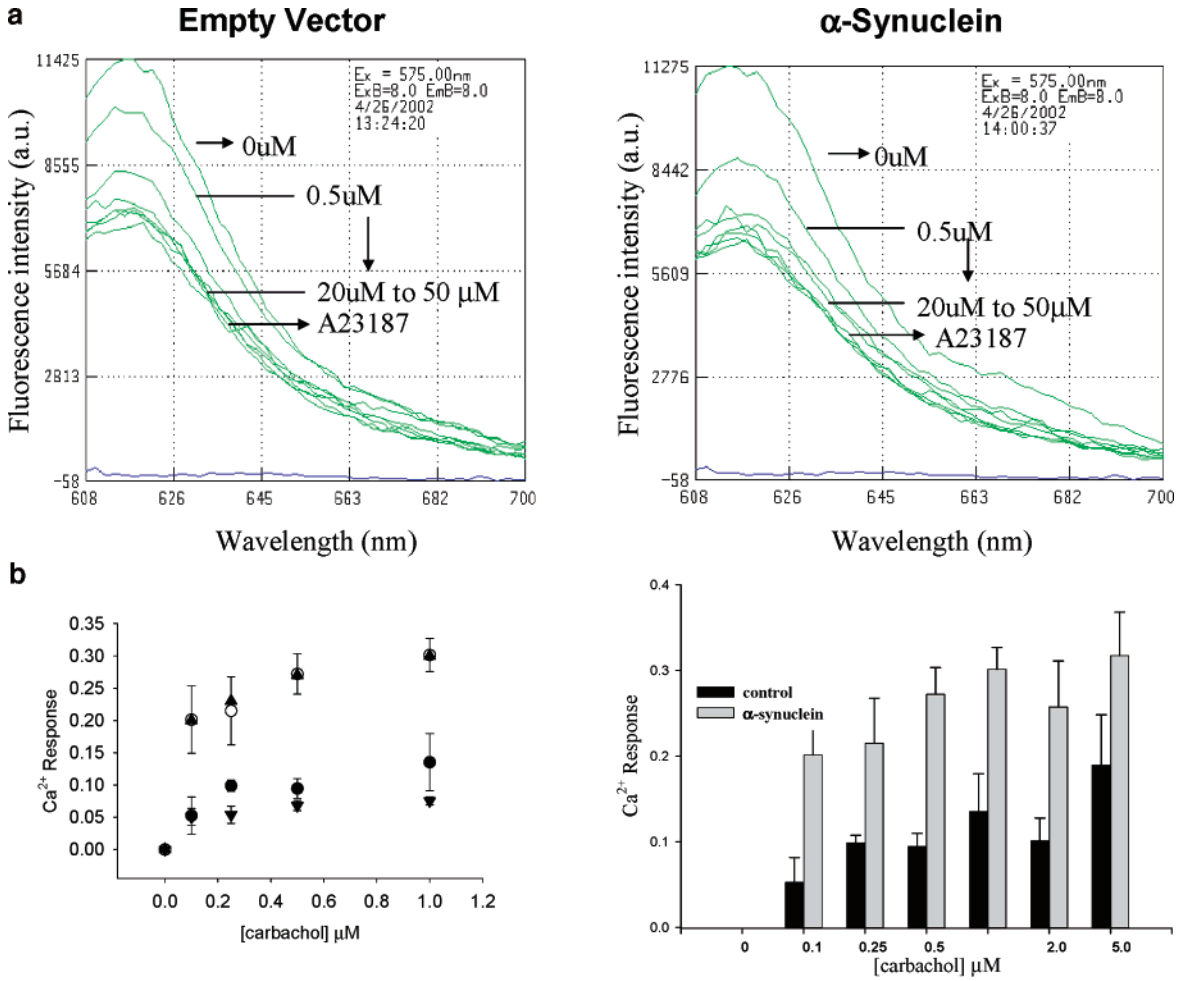

FIGURE 4: α-Synuclein enhances the Ca^{2+} response to carbachol in A10 cells. (a) Raw (i.e., uncorrected) spectra showing the change in intensity of Calcium Crimson upon carbachol stimulation of A10 cells transfected with the empty vector or α-synuclein. Carbachol concentrations are noted in the figure. Also shown are the spectra when the Ca^{2+} ionophore A23187 is added, which would allow the entry of extracellular Ca^{2+} into the cell up to the concentration in the buffer (0.67 mM free Ca^{2+}). (b) Change in fluorescence intensity (FI) of Calcium Crimson in A10 cells transfected with either α-synuclein or the empty vector, where the Ca^{2+} response (*Y*-axis) is calculated as 1 − FI(α-synuclein)/FI(control). The data are an average of four independent experiments carried out in triplicate, and the error was calculated using SigmaStat. In the left panel of part b is shown the carbachol response of control cells (▼), PTX-treated α-synuclein-transfected cells (●) ($n = 3$), and α-synuclein-transfected cells in the presence and absence of extracellular calcium ($n = 3$) (○ and ▲). In the right panel are shown the data from the left panel replotted to allow for direct comparison of the Ca^{2+} response of control and α-synuclein-transfected cells.

but localized mainly on the plasma membrane and not in the nucleus (*19*, *25*, *38*−*40*).

We measured the increase in cellular Ca^{2+} concentration by labeling cells with a fluorescent Ca^{2+} indicator (Calcium Crimson) and then stimulating them with G protein-coupled receptor agonists. In A10 cells, Calcium Crimson is sequestered in the endoplasmic reticulum, and release of Ca^{2+} from the endoplasmic reticulum upon cell stimulation results in a decrease in the fluorescence intensity (see ref *41*). While results identical to those described below were obtained using Fura-2AM, we found Calcium Crimson was less toxic to the cells.

Studies were carried out by adding either carbachol or acetylcholine to a suspension of Calcium Crimson-loaded A10 cells while stirring and immediately recording the fluorescence spectra. We present data for studies using carbachol and note that identical results were obtained using acetylcholine (ACh) at similar concentrations. An example of the uncorrected spectral changes is shown in Figure 4a. From the spectra, we find that the intensity change upon going from 0 to 0.5 μM carbachol is much greater in the cells transfected with α-synuclein than with the empty vector. A compilation of these studies is shown in Figure 4b, where we present the ratio of the rate of Ca^{2+} release of α-synuclein-transfected cells to that of empty vector-transfected cells as a function of increasing carbachol concentrations. These data thus represent the extent to which α-synuclein-transfected cells are more responsive to agonists than the control vector-transfected cells.

The extent of the increase in Ca^{2+} concentration in the α-synuclein-expressing cells depended on the amount of agonist added. With small amounts of added carbachol (0.1 μM), cells expressing α-synuclein exhibited a 4-fold increase in Ca^{2+} concentration over control cells (Figure 4b). If one considers that the transfection efficiency was only ∼50−80%, then this response corresponds to an as much as ∼6−8-fold increase in the rate of Ca^{2+} release. At higher carbachol concentrations where responses should saturate on the basis of analogy with a closely related cell line (*42*), the Ca^{2+} response of A10 cells with α-synuclein became more comparable to that of the cells transfected with the empty vector. These results suggest that the major effect of

α-synuclein occurs at low levels of agonist. Similar results were obtained using another fluorescent calcium indicator, Fura2-AM, indicating that this response is independent of the probe used for the Ca^{2+} measurements (data not shown).

The increase in Ca^{2+} levels seen in the α-synuclein-transfected cells could be due to the release of Ca^{2+} from intracellular stores or due to the entry of extracellular calcium from the opening of Ca^{2+} channels (see ref *43*). To determine whether entry of extracellular Ca^{2+} is contributing to the results depicted in Figure 4, we repeated the study described above by washing and dispersing the cells in a Ca^{2+}-free buffer (i.e., no added Ca^{2+} and 1 mM EGTA) so that entry of extracellular Ca^{2+} cannot occur. The data collected in these studies were the same within error as the data in Figure 4b, suggesting that the increased Ca^{2+} levels are due to release from intracellular stores.

Stimulation of cells by carbachol activates both the $G\alpha_q$ and $G\alpha_i$ families, and PLCβ_2 could be activated by either family. $G\alpha_q$ subunits directly activate PLCβ_2, while $G\alpha_i$ subunits release G$\beta\gamma$ subunits which activate PLCβ_2. Thus, both pathways may result in an increase in cellular Ca^{2+}. To determine the contribution of $G\alpha_q$ to the results depicted in Figure 4b, we pretreated the cells with pertussis toxin (PTX). This agent selectively eliminates stimulation of $G\alpha_i$ subunits. PTX treatment greatly reduced the extent of enhancement of Ca^{2+} in the α-synuclein-transfected cells upon stimulation (Figure 4b), suggesting that $G\alpha_i$ and their associated G$\beta\gamma$ subunits are responsible for the effect of α-synuclein on intracellular Ca^{2+} release, and that the *in vitro* enhancement of G$\beta\gamma$−PLCβ_2 activity by α-synuclein may occur, in part, in cells. The effect of PTX was quantified further by assessing inositol phosphate production as described below.

α-Synuclein Enhances Inositol Phosphate Production in Cultured Cells. The Ca^{2+} studies suggest that α-synuclein may be involved in inositol phospholipid signaling. To better determine whether α-synuclein could affect PLCβ_2 activity, we monitored the production of inositol phosphates (InsP) which is more directly related to PLC activity than downstream Ca^{2+} levels. We measured the amount of [^{3}H]InsP generated after agonist stimulation as described in Experimental Procedures (see ref *31*) and found that stimulation of untransfected wild-type A10 cells by either ACh (1 μM) or carbachol (1 μM) gave a similar 1.5−2-fold increase in the amount of [^{3}H]InsP, in accord with other reports (Figure 5a) (*44*). We note that basal levels of [^{3}H]InsP in cells transfected with α-synuclein were consistently 12 ± 7% lower than those of cells transfected with the empty vector which could be due to PLCβ_2 inhibition by α-synuclein (see the Discussion). However, cells transfected with α-synuclein showed a statistically larger increase (i.e., ∼1.4-fold) in the level of [^{3}H]InsP production with carbachol than with cells transfected with the empty vector (Figure 5b). Thus, the presence of α-synuclein allows the carbachol-induced level of [^{3}H]InsP to increase 2.8-fold. Importantly, the increase in [^{3}H]InsP levels with α-synuclein-expressing A10 cells was eliminated by pretreatment with PTX (Figure 5b), strongly suggesting that the increase observed with α-synuclein may result from PLCβ_2 activation by G$\beta\gamma$ subunits released from $G\alpha_i$. Also, addition of 1 μM insulin or 100 ng/mL epidermal growth factor (EGF) did not result in a detectable increase in the level of [^{3}H]InsP production in α-synuclein-expressing cells versus control cells, indicating that α-synuclein is not mediating inositol phosphate levels through receptor tyrosine kinase−PLCγ pathways (Figure 5c).

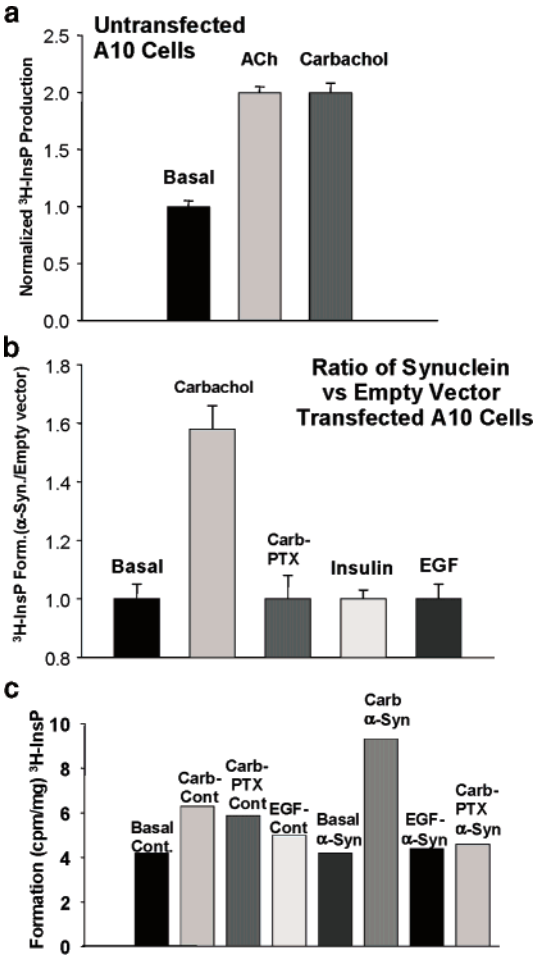

FIGURE 5: α-Synuclein enhances [^{3}H]InsP production in A10 cells. (a) Total increase in the level of [^{3}H]InsP in untransfected cells with the addition of 1 μM ACh or 1 μM carbachol. (b) Increase in the level of [^{3}H]InsP in α-synuclein A10-transfected cells divided by cells transfected with empty vector with the addition of 1 μM carbachol, 1 μM insulin, or 100 ng/mL EGF in untreated and PTX-treated cells. (c) Level of radioactivity in counts per minute per milligram of protein of a single set of studies where Cont refers to cells transfected with the empty vector and α-Syn refers to cells transfected with α-synuclein. [^{3}H]Ins(1,4,5)P_3 production was assessed as described in Experimental Procedures, where $n = 6-9$ except where noted. Average values with standard errors are given and were calculated using SigmaStat.

Although α-synuclein is expressed in various tissues to some extent, it is found at high levels in neuronal cell lines (*45*, *46*). We then repeated the studies depicted in Figure 5 in the neuronal cell line PC12, which exhibits a neuronal phenotype after pretreatment with nerve growth factor. Using Western blot analysis, we confirmed previous studies suggesting that these cells contain endogenous α-synuclein and PLCβ_2 (refs *47* and *48* and data not shown).

Using a similar methodology as described above, we found that when PC12 cells were transfected with α-synuclein they displayed a ∼40% decrease in the basal [^{3}H]InsP level relative to control cells (e.g., Figure 6, top) which may again correlate with a reduced PLCβ activity. However, α-synuclein-transfected cells exhibited an ∼1.6-fold enhancement with carbachol over controls (Figure 6, bottom). PTX treatment eliminated the carbachol-induced increase, again

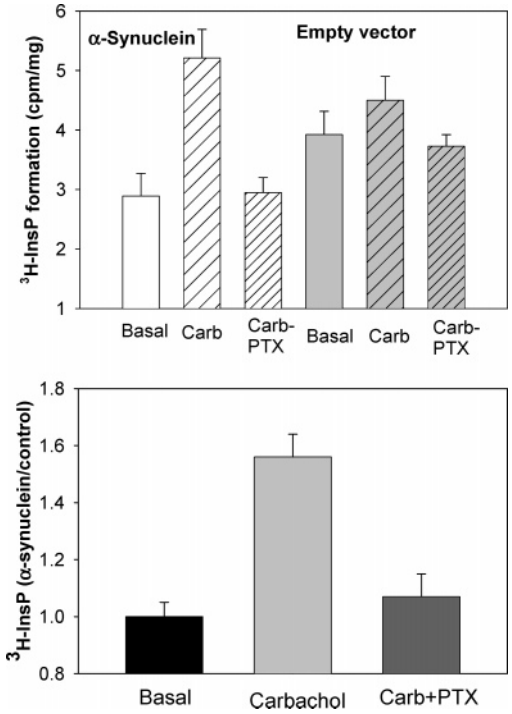

FIGURE 6: α-Synuclein enhances [^{3}H]InsP production in PC12 cells. This figure presents a study similar to that depicted in Figure 5 using the neural-like cell line, PC12, where the top panel shows the radioactivity counted for a single set of samples and the bottom panel shows the normalized ratio of the response of α-synuclein transfected to that of the empty vector ($n = 6$).

indicating a large contribution of G$\beta\gamma$ subunits released by Gα_i. These results show the involvement of α-synuclein in inositol phosphate homeostasis in cultured cells.

DISCUSSION

α-Synuclein has been linked to both intracellular dopamine signaling and Ca^{2+} regulation. The coupling of PI(4,5)P_2, PLCβ_2, and G$\beta\gamma$ to Ca^{2+} and dopamine signals is well-established (*24*−*27*). Here, we present data suggesting that α-synuclein prefers membranes containing the signaling lipid PI(4,5)P_2 and regulates activation of PLCβ_2 by G$\beta\gamma$. These results suggest a link between α-synuclein and the inositol phospholipid signaling pathway. PLCβ_2−G$\beta\gamma$ regulation by α-synuclein was directly shown using purified proteins and model membranes, and indicated in cells by measuring increases in the levels of the PLC product, inositol phosphate, and a more downstream PLC product, intracellular Ca^{2+}, under conditions where activation of PLCβ_2 by G$\beta\gamma$ occurs.

While the magnitude of Ca^{2+} release and [^{3}H]InsP production can be influenced by many cellular factors, our *in vitro* studies clearly showed that α-synuclein has a preference for membranes containing PI(4,5)P_2 membranes, and that it binds to the PI(4,5)P_2-hydrolyzing enzyme PLCβ_2 with high affinity. More so, the binding affinity for PI(4,5)P_2-containing membranes can be further enhanced by the addition of high levels of Ca^{2+}. The concentrations of Ca^{2+} used in the membrane binding studies were chosen from titrations that followed the loss in fluorescence intensity α-synuclein as Ca^{2+} or Mg^{2+} was added (Figure 1b). The loss in intrinsic fluorescence intensity with increased Mg^{2+} may be attributed to the quenching as the cation weakly associates with the acidic C-terminus. The higher degree of quenching by Ca^{2+} versus Mg^{2+} suggests that α-synuclein interacts with Ca^{2+} with a higher affinity. Previous studies by Nielsen and co-workers (*32*) have identified a novel Ca^{2+} binding motif in the C-terminus whose weak Ca^{2+} affinity, more than 1 order of magnitude higher than physiological Ca^{2+} levels (IC_{50} = 300 μM), suggests a role in nerve terminals or in regions surrounding Ca^{2+} channels. Our studies suggest that very high Ca^{2+} levels will better stabilize α-synuclein membrane localization and subsequent association with membrane-associated proteins. Alternately, the weak apparent Mg^{2+} association as compared with the free concentration of 0.5 mM in cells may not play a functional role.

Since α-synuclein is an unstructured protein with chaperone capabilities, it is not completely surprising that it binds strongly and influences the activity of PLCβ_2. However, this interaction is unusually strong (i.e., K_d < 1 nM), and the ability of α-synuclein to specifically inhibit PLCβ_2 and not PLC$\beta_{1,3}$ is unexpected (see below). It is also very surprising that α-synuclein is able to enhance the stimulation of PLCβ_2 by G$\beta\gamma$ from ∼4- to ∼24-fold and that α-synuclein only affects PLCβ_2 activation by G$\beta\gamma$ and not by Gα_q subunits. There are several mechanisms that may be responsible for this behavior. First, α-synuclein may direct the membrane binding of PLCβ_2 due to its strong membrane binding affinity, but we note that the enzymatic assays were carried out at lipid concentrations much higher than the membrane partition coefficient of PLCβ_2 (*22*), so this cannot be the case for the observed *in vitro* results. Alternately, α-synuclein may either directly or indirectly promote the conformational changes in the catalytic site of PLCβ_2 that G$\beta\gamma$ confers during activation. However, α-synuclein did not affect the concentration dependence of the PLCβ_2 activation curve by G$\beta\gamma$, suggesting that the action of α-synuclein is not to promote association between PLCβ_2 and G$\beta\gamma$. More studies are currently underway to understand the structure that α-synuclein adapts when bound to PLCβ_2 to allow for these dramatic changes in G$\beta\gamma$ protein activation. It is important to note that since α-synuclein is found in high concentrations in some cell lines, it is quite possible that it influences the activity and stimulation of other cellular enzymes.

The A53T mutation of α-synuclein has been associated with familial Parkinson's disease (*9*). This conservative mutation was only half as potent in reducing the enhancement of G$\beta\gamma$ activation of PLCβ_2 compared to the wild type. While it is unclear whether this reduction in activation could lead to pathological states, these data unexpectedly show that a small, conservative mutation can have significant functional effects in unstructured proteins such as α-synuclein.

We extended the *in vitro* studies to determine whether it was possible that α-synuclein affects PLCβ_2 activation by G$\beta\gamma$ subunits in cells. Initially, we find that the presence of α-synuclein results in an at least 4-fold increase over control cells in the rate of release of intracellular Ca^{2+} in A10 cells. We also find that α-synuclein reduces basal levels of [^{3}H]-InsP and whether this reduction is due to its inhibition of PLCβ activity or other factors that affect the inositol lipid pool is unclear at the moment. It is clear that α-synuclein allows for a much higher level of stimulation upon addition of an agonist. This can be compared to the 1.7−2.0-fold increase in the level of InsP and a 3.6−3.8-fold increase in Ca^{2+} levels seen when A10 cells are stimulated with arginine vasopressin or endothelin (*44*, *49*), suggesting that the

presence of α-synuclein can have the effect of a second stimulator.

Ca^{2+} and inositol phosphate levels in cells are tightly regulated, and it was surprising to observe that α-synuclein was able to increase their stimulated levels. It is noteworthy that PLCϵ enzymes can also be activated by G protein subunits (*50*−*52*), but we have not as yet determined their contribution to Ca^{2+} and InsP levels, or their possible interaction with α-synuclein. Regulation of PLCϵ by α-synuclein should be the subject of further study. It is also noteworthy that PLCδ enzymes, which are expressed in both cell lines and have a high specific activity, are activated with increases in basal Ca^{2+} levels and lie downstream from other PLCs (see ref *22*), and it is likely that PLCδ enzymes may contribute to the Ca^{2+} and InsP responses reported here. Thus, while it is temping to speculate that the effects we observe here are due directly to interaction of α-synuclein with PLCβ_2, it is probable that other synergistic mechanisms are operative.

Our *in vitro* studies show that α-synuclein specifically enhances PLCβ_2 activation by G$\beta\gamma$ subunits and not $G\alpha_q$. Since G$\beta\gamma$ subunits are generally released upon activation of many G protein-coupled receptors, there is the potential for enhanced Ca^{2+} release by α-synuclein through PLCβ_2 upon treatment with a wide range of agonists. Carbachol will stimulate both $G\alpha_q$ and $G\alpha_i$ subunits, and G$\beta\gamma$ release from either family could potentially activate PLCβ_2. PTX treatment allowed us to assess the contribution of $G\alpha_q$ from $G\alpha_i$, and the contribution of the G$\beta\gamma$ subunits released from these two G protein families. We find that in both cell lines, G$\beta\gamma$ subunits released from $G\alpha_i$ subunits and not $G\alpha_q$ contribute to the α-synuclein enhancement of PLCβ_2. Of course, other cell lines may differ depending on the expression and localization of muscarinic receptors and G protein subunits.

In neuronal-like PC12 cells, the level of expression of PLCβ_2 is much lower as compared to those of other PLCβ isoforms (*47*, *48*), and we find by Western blot analysis low but detectable levels of PLCβ_2 in both primary cortical neurons and PC12 cells (data not shown). We also note that preliminary secondary immunofluorescence images indicate colocalization of endogenous PLCβ_2 and α-synuclein in PC12 cells and colocalization of these proteins in transfected A10 cells (data not shown). The results presented here suggest that, among other possible mechanisms, the interaction of PLCβ_2 with α-synuclein may allow it to contribute to Ins(1,4,5)P_3 production upon G protein stimulation and compensate for its lower level of expression as compared to the more prevalent PLCβ_1, or the more active downstream enzyme PLCδ.

It is interesting to note that soon after the first isolation of α-synuclein from cholinergic vesicles (*53*), the distribution of rat brain α-synuclein was found to closely parallel that of PLCβ, m1, and m3 muscarinic cholinergic receptors and Gα subunits, which are all major players in the phosphoinositide signaling pathway. Also, it has recently been shown using photo-cross-linking that α-synuclein binds to calmodulin in a Ca^{2+}-dependent manner with an apparent affinity of 20 nM (*54*). Although the affinity of α-synuclein and PLCβ_2 estimated here is quite strong, due to its abundance in neural cells, α-synuclein is expected to interact with other partners as well. α-Synuclein has already been found to associate with unknown affinities with a variety of proteins, including tau (*55*), extracellular regulated kinase (*56*), and phopholipase D (*15*), and in some cases inhibiting their activities. We find that α-synuclein also inhibits PLCβ_2 which may attenuate the basal Ins(1,4,5)P_3 contribution of PLCβ_2 in the absence of G protein agonists. The large number of binding partners most likely stems from the observation that α-synuclein purifies as a unfolded protein (*16*), can act as a molecular chaperone (*14*), and can bind strongly to membranes which would promote its interaction with membrane proteins. These various interactions most likely serve to prevent aggregation and fibril formation (*18*, *20*, *21*, *57*).

The results from this study suggest that α-synuclein may be linked to Ca^{2+} regulation through phospholipase Cβ. Since Ca^{2+} regulation is in turn linked to a host of cellular events, including vesicle trafficking, dopamine uptake, and receptor sequestration, the interconnection between these events may provide clues to the precise cellular function of α-synuclein.

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REFERENCES

1. Dawson, T., Mandir, A., and Lee, M. (2002) Animal models of PD: Pieces of the same puzzle? *Neuron 35*, 219−222.
2. Duda, J. E., Lee, V. M., and Trojanowski, J. Q. (2000) Neuropathology of synuclein aggregates, *J. Neurosci. Res. 61*, 121−127.
3. Ueda, K., Fukushinma, F., Masliah, E., et al. (1993) Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer's disease, *Proc. Natl. Acad. Sci. U.S.A. 90*, 11282−11286.
4. Hashimoto, M., Hsu, L. J., Sisk, A., Xia, Y., Takeda, A., et al. (1998) Human recombinant NACP/α-synuclein is aggregated and fibrillated in vitro: Relevance for Lewy body disease, *Brain Res. 799*, 301−306.
5. Clayton, D. F., and George, J. M. (1998) The synucleins: A family of proteins involved in synaptic function, plasticity, neurodegeneration and disease, *Trends Neurosci. 21*, 249−254.
6. Lavedan, C. (1998) The synuclein family, *Genome Res. 8*, 871−880.
7. Clayton, D. F., and George, J. M. (1999) Synucleins in synaptic plasticity and neurodegenerative disorders, *J. Neurosci. Res. 58*, 120−129.
8. Burke, R. E. (1999) α-Synuclein and Parkinson's disease, *Brain Res. Bull. 50*, 465−466.
9. Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S., Dehejia, A., et al. (1997) Mutation in the α-synuclein gene identified in families with Parkinson's disease, *Science 276*, 2045−2047.
10. Kruger, R., Muller, T., and Riess, O. (2000) Involvement of α-synuclein in Parkinson's disease and other neurodegenerative disorders, *J. Neural Transm. 107*, 31−40.
11. Lee, F. J., et al. (2001) Direct binding and functional coupling of α-synuclein to the dopamine transporters accelerate dopamine-induced apoptosis, *FASEB J. 15*, 916−926.
12. Lotharius, J., and Brundin, P. (2002) Impaired dopamine storage resulting from α-synuclein mutation may contribute to the pathogensis of Parkinson's disease, *Hum. Mol. Genet. 11*, 2395−2407.
13. George, J. M. (2002) The synucleins, *Genome Biol. 3*, 3002.1−3002.6.
14. Ostrerova, N., Petrucelli, L., Farrer, M., Mehta, N., Choi, P., et al. (1999) α-Synuclein shares physical and functional homology with 14-3-3 proteins, *J. Neurosci. 19*, 5782−5791.
15. Jenco, J. M., Rawlingson, A., Daniels, B., and Morris, A. J. (1998) Regulation of phospholipase D2: Selective inhibition of mammalian phospholipase D isoenzymes by α- and β-synucleins, *Biochemistry 37*, 4901−4909.

16. Weinreb, P. H., Zhen, W., Poon, A. W., Conway, K. A., and Lansbury, P. T., Jr. (1996) NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded, *Biochemistry 35*, 13709−13715.
17. Conway, K. A., Lee, S. J., Rochet, J. C., Ding, T. T., Williamson, R. E., and Lansbury, P. T., Jr. (2000) Acceleration of oligomerization, not fibrillization, is a shared property of both α-synuclein mutations linked to early-onset Parkinson's disease: Implications for pathogenesis and therapy, *Proc. Natl. Acad. Sci. U.S.A. 97*, 571−576.
18. Davidson, W. S., Jonas, A., Clayton, D. F., and George, J. M. (1998) Stabilization of α-synuclein secondary structure upon binding to synthetic membranes, *J. Biol. Chem. 273*, 9443−9449.
19. Lee, H. J., Choi, C., and Lee, S. J. (2002) Membrane-bound α-synuclein has a high aggregation propensity and the ability to seed the aggregation of the cytosolic form, *J. Biol. Chem. 277*, 671−678.
20. Narayanan, V., and Scarlata, S. (2001) Membrane binding and self-association of α-synuclein, *Biochemistry 40*, 9927−9934.
21. McLean, P. J., Kawamata, H., Ribich, S., and Hyman, B. T. (2000) Membrane association and protein conformation of α-synuclein in intact neurons. Effect of Parkinson's disease-linked mutations, *J. Biol. Chem. 275*, 8812−8816.
22. Rebecchi, M. J., and Pentyala, S. N. (2000) Structure, function, and control of phosphoinositide-specific phospholipase C, *Physiol. Rev. 80*, 1291−1335.
23. Rhee, S. G. (2001) Regulation of phosphoinositide-specific phospholipase C, *Annu. Rev. Biochem. 70*, 281−312.
24. Rasolonjanahary, R., Gerard, C. M. N. D., Homburger, V., Enjalbert, A., and Guillon, G. (2002) Evidence for a direct negative coupling between dopamine-D2 receptors and PLC by heterotrimeric Gi1/2 in rat anterior pituitary cell membranes, *Endocrinology 143*, 747−754.
25. Stefanis, L., Kholodilov, N., Rideout, H. G., Burke, R. E., and Greene, L. A. (2001) Synuclein-1 is selectively up-regulated in response to nerve growth factor treatment in PC12 cells, *J. Neurochem. 76*, 1165−1176.
26. Abeliovich, A., Schmitz, Y., Farinas, I., Choi-Lundberg, D., Ho, W. H., et al. (2000) Mice lacking α-synuclein display functional deficits in the nigrostriatal dopamine system, *Neuron 25*, 239−252.
27. LaFerla, F. (2002) Calcium dyshomeostasis and intracellular signalling in Alzheimer's disease, *Nat. Rev. Neurosci. 3*, 862−872.
28. Park, S. M., Jung, H. Y., Rhim, H., Paik, S. R., Chung, K. C., et al. (2002) Evidence that α-synuclein functions as a negative regulator of Ca^{2+}-dependent α-granule release from human platelets, *Blood 100*, 2506−2514.
29. Runnels, L., and Scarlata, S. (1999) Determination of the affinities between heterotrimeric G protein subunits and their phospholipase C-β effectors, *Biochemistry 38*, 1488−1496.
30. Wang, T., Pentyala, S., Rebecchi, M. J., and Scarlata, S. (1999) Differential association of the pleckstrin homology domains of phospholipases C-β1, C-β2, and C-δ1 with lipid bilayers and the $\beta\gamma$ subunits of heterotrimeric G proteins, *Biochemistry 38*, 1517−1524.
31. Weernink, P. A., Guo, Y., Zhang, C., Schmidt, M., Von Eichel-Streiber, C., and Jakobs, K. H. (2000) Control of cellular phosphatidylinositol 4,5-bisphosphate levels by adhesion signals and rho GTPases in NIH 3T3 fibroblasts involvement of both phosphatidylinositol-4-phosphate 5-kinase and phospholipase C, *Eur. J. Biochem. 267*, 5237−5246.
32. Nielsen, M. S., Vorum, H., Lindersson, E., and Jensen, P. H. (2001) Ca^{2+} binding to α-synuclein regulates ligand binding and oligomerization, *J. Biol. Chem. 276*, 22680−22684.
33. Wang, T., Dowal, L., El-Maghrabi, M. R., Rebecchi, M., and Scarlata, S. (2000) The pleckstrin homology domain of phospholipase C-β_2 links the binding of G$\beta\gamma$ to activation of the catalytic core, *J. Biol. Chem. 275*, 7466−7469.
34. Boyer, J. L., Graber, S. G., Waldo, G. L., Harden, T. K., and Garrison, J. C. (1994) Selective Activation of Phospholipase C by Recombinant G-protein α- and $\beta\gamma$-Subunits, *J. Biol. Chem. 269*, 2814−2819.
35. Scarlata, S., Ed. (2002) *Determination of the strength and specificity of membrane-bound protein association using fluorescence spectroscopy: Application to the G protein-phospholipase C system*, Vol. 345, Academic Press, New York.
36. Wach, L., Brink, C., and Norel, X. (2001) The muscarinic receptor subtypes in human blood vessels, *Therapie 3*, 223−226.
37. Di Salvo, J., and Nelson, S. R. (1998) Stimulation of G-protein coupled receptors in vascular smooth muscle cells induces tyrosine kinase dependent increases in calcium without tyrosine phosphorylation of phospholipase C γ-1, *FEBS Lett. 422*, 85−88.
38. Hashimoto, M., et al. (1997) NACP, a synaptic protein involved in Alzheimer's disease, is differentially regulated during megakaryocyte differentiation, *Biochem. Biophys. Res. Commun. 237*, 611−616.
39. Zhou, W., Hurlbert, M. S., Schaack, J., Prasad, K. N., and Freed, C. R. (2000) Overexpression of human α-synuclein causes dopamine neuron death in rat primary culture and immortalized mesencephalon-derived cells, *Brain Res. 866*, 33−43.
40. Takenouchi, T., et al. (2001) Reduced neuritic outgrowth and cell adhesion in neuronal cells transfected with human α-synuclein, *Mol. Cell. Neurosci. 17*, 141−150.
41. Takahashi, A. (1999) Measurement of intracellular calcium, *Physiol. Rev. 49*, 189.
42. Kobayashi, S., Ikeda, K., Miyata, K., Yamada, T., and Honda, K. (2001) A method for measurement of muscarinic receptor-mediated responses in dissociated single colon longitudinal smooth muscle cells, *J. Pharmacol. Toxicol. Methods 45*, 199−205.
43. Berridge, M. J., Lipp, P., and Bootman, M. D. (2000) The versatility and universality of calcium signaling, *Nat. Rev. Mol. Cell Biol. 1*, 11−21.
44. Xuan, Y.-T., Whorton, A. R., and Watkins, W. D. (1989) Inhibition by nicrardipine on endothelin-mediated inositol phosphate formation and Ca^{2+} mobilization in smooth muscle cell, *Biochem. Biophys. Res. Commun. 160*, 758−764.
45. Maroteaux, L., and Scheller, R. H. (1991) The rat brain synucleins: Family of proteins transiently associated with neuronal membrane, *Brain Res. Mol. Brain Res. 11*, 335−343.
46. Marui, W., Iseki, E., Ueda, K., and Kosaka, K. (2000) Occurrence of human α-synuclein immunoreactive neurons with neurofibrillary tangle formation in the limbic areas of patients with Alzheimer's disease, *J. Neurol. Sci. 174*, 81−84.
47. Mizuguchi, M., Yamada, M., Kim, S. U., and Rhee, S. G. (1991) Phospholipase C isozymes in neurons and glial cells in culture: An immunocytochemical and immunochemical study, *Brain Res. 548*, 35−40.
48. Gerfen, C. R., Choi, W. C., Suh, P. G., and Rhee, S. G. (1988) Phospholipase C I and II brain isozymes: Immunohistochemical localization in neuronal systems in rat brain, *Proc. Natl. Acad. Sci. U.S.A. 85*, 3208−3212.
49. Ishibashi, T., Yoshida, Y., Imai, S., and Nishio, M. (1999) IP3 production in A10 cells, an established aortic smooth muscle cultured cell line: Effects of agonist administration procedure and culture conditions, *Gen. Pharmacol. 32*, 115−121.
50. Lopez, I., Mak, E. C., Ding, J., Hamm, H. E., and Lomasney, J. W. (2001) A novel bifunctional phospholipase C that is regulated by Gα 12 and stimulates the Ras/mitogen-activated protein kinase pathway, *J. Biol. Chem. 276*, 2758−2765.
51. Wing, M., Houstaon, D., Kelley, G., Der, C., Siderovski, D., and Harden, K. (2001) Activation of phospholipase C-ϵ by heterotrimeric G protein $\beta\gamma$ subunits, *J. Biol. Chem. 276*, 48257−48251.
52. Kelley, G. G., Reks, S. E., and Smrcka, A. V. (2004) Hormonal regulation of phospholipase Cϵ through distinct and overlapping pathways involving G12 and Ras family G-proteins, *Biochem. J. 378*, 129−139.
53. Maroteaux, L., Campanelli, J. T., and Scheller, R. H. (1988) Synuclein: A neuron-specific protein localized to the nucleus and presynaptic nerve terminal, *J. Neurosci. 8*, 2804−2815.
54. Martinez, J., Moeller, I., Erdjument-Bromage, H., Tempst, P., and Lauring, B. (2003) Parkinson's disease-associated α-synuclein is a calmodulin substrate, *J. Biol. Chem. 278*, 17379−17387.
55. Jensen, P. H., Islam, K., Kenney, J., Nielsen, M. S., Power, J., and Gai, W. P. (2000) Microtubule-associated protein 1B is a component of cortical Lewy bodies and binds α-synuclein filaments, *J. Biol. Chem. 275*, 21500−21507.
56. Iwata, A., Miura, S., Kanazawa, I., Sawada, M., and Nukina, N. (2001) α-Synuclein forms a complex with transcription factor Elk-1, *J. Neurochem. 77*, 239−252.
57. Jo, E., McLaurin, J., Yip, C. M., St George-Hyslop, P., and Fraser, P. E. (2000) α-Synuclein membrane interactions and lipid specificity, *J. Biol. Chem. 275*, 34328−34334.

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