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α -Synuclein Increases the Cellular Level of Phospholipase C β 1

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Abstract

α -Synuclein is a conserved protein that is a key component in neurodegenerative plaques [1, 2]. α -Synuclein binds strongly to phospholipase C β (PLC β) and promotes Ca^{2+} release in cells. Here, we show that expression of α -synuclein increases the cellular level of PLC β 1 in two neuronal cell lines: PC12 and SK-N-SH. The increase in PLC β 1 is not accompanied by changes in the level of RNA or in ubiquitination. Instead, we find that α -synuclein protects PLC β 1 from trypsin digestion and from degradation by the Ca^{+2} activated protease calpain. Calpain removes the C-terminal region of the enzyme which mediates activation by $\text{G}\alpha_q$. We find that in SK-N-SH cells, α -synuclein reduced degradation of PLC β 1 by calpain during Ca^{2+} signaling allowing the enzyme to remain sensitive to $\text{G}\alpha_q$ activation. Taken together, our studies show that α -synuclein protects the integrity of PLC β 1 and its ability to be activated by $\text{G}\alpha_q$, which may in turn impact Ca^{2+} signaling.

1 INTRODUCTION

α -Synuclein is a small protein that is highly expressed in brain tissues (see [1]). α -Synuclein is the major component of neurodegenerative plaques and mutations are associated with sporadic forms of familial Parkinson's disease [5–8]. This association with neurodegenerative disorders has sparked much interest in the physical and cellular properties of the protein. To date, α -synuclein has been found only in vertebrates and is highly conserved from humans to songbirds [2]. This high degree of conservation suggests an important role in the cell although its function is still unknown.

α -Synuclein binds to many proteins (see [3]) and also to lipid membranes (e.g.[10–13]). Studies using knock-out mice point to a role in neural plasticity responses [14], and show a connection between α -synuclein and ApoE and A β peptides [4], suggesting a role in lipid trafficking. It is notable that many of α -synuclein potential partners are involved in lipid signaling pathways (see [5]).

We have previously found that membrane binding of α -synuclein is promoted by PI(4,5)P $_2$ and Ca^{2+} suggesting that it might be involved in the PLC signaling pathway [6]. PLC β enzymes are activated by the $\text{G}\alpha_q$ family of G proteins in response to specific neurotransmitters and hormones. PLC β s catalyze the hydrolysis of PI(4,5)P $_2$ to generate the release of Ca^{2+} from intracellular stores and activate protein kinase C. There are four known types of PLC β with varying tissue distribution and sensitivity to G proteins. PLC β 1 is the

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most sensitive to $G\alpha_q$ and has the least sensitivity to $G\beta\gamma$ subunits. In contrast, PLC β 2 and PLC β 3 are sensitive to both $G\alpha_q$ and $G\beta\gamma$ subunits.

Our previous study found that α -synuclein associates with PLC β 1–3 with a high affinity reducing their basal activity by ~50% [6]. This association strongly enhanced activation by $G\beta\gamma$ subunits although it did not appear to affect activation by $G\alpha_q$ subunits [6]. We have extended those studies to include interactions between α -synuclein and PLC β 1, which are both highly expressed in neuronal cells. In the course of these studies, we observed that as the cellular level of α -synuclein increases, so does the level of PLC β 1, but not vice versa. This observation led to a series of experiments showing that α -synuclein protects the enzyme from calpain digestion and preserves regulation by $G\alpha_q$. Our studies show α -synuclein that can serve as a protective agent that may potentially impact the cell signaling. The results also imply that removal of one or more protein partners will promote self-association and aggregation of α -synuclein.

2 MATERIALS AND METHODS

2.1 Cell culture and transfection

HEK TAP-PLC β 1 cells were a generous gift from Dr. Loren Runnels (UMDNJ Piscataway, NJ) and have been described [7]. SK-N-SH cells and PC12 cells were obtained from ATCC (HTB-11, CRL-1721). Both HEK TAP-PLC β 1 and SK-N-SH cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO 11965) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μ g/ml streptomycin sulfate. PC12 cells were cultured in DMEM medium supplemented with 10% horse serum, 5% fetal bovine serum, 50 units/ml penicillin, and 50 μ g/ml streptomycin sulfate. All cells were incubated at 37 °C with 5% CO₂. Tetracycline was added to a final concentration 1 μ g/ml to induce PLC β 1 expression in HEK TAP-PLC β 1 cells. Nerve growth factor (NGF, Sigma, St. Louis, MO) was added to a final concentration of 100 ng/ml to induce PC12 cells differentiation. Plasmids were introduced into cells grown at 80–90% confluency by electroporation for PC12 cells (<http://www.sciencedirect.com/science/article/pii/S0003986111000701-b0030>) and calcium phosphate precipitation for HEK TAP-PLC β 1 cells. The protein expression of endogenous α -synuclein in SK-N-SH cells was knocked down using small interference RNA α -synuclein (Dharmacon Inc.) according to the manufacturer's instructions along with the negative control purchased from the manufacturer. The cells were incubated for 72 hours post knock down at 37°C in 5% CO₂. Western blot analysis showed this procedure produces ~80% gene silencing.

2.2 Protein purification

His₆-PLC β 1 and PLC β 2 were expressed in Sf9 cells using a baculovirus system with minor modifications [17]. A C-terminal truncation mutant of PLC β 2 using for some of the control studies is actually a chimeric construct of PLC β 2 and PLC δ 1 described in [8]. α -Synuclein, γ -synuclein and the PH β 2/PLC δ 1 chimera were expressed in *Escherichia coli* and purified on a Ni²⁺ column using previously reported methods (see [9]). Expression and purity was assessed by Western blot analysis using commercial antibodies, purchased from Abcam (α -synuclein and γ -synuclein), and Santa Cruz Biochemicals (PLC β 1).

2.3 Enzyme digestion experiments

Digestion studies used purified proteins and were carried out by western blot analysis using anti- PLC β 1 (Santa Cruz Biochemicals sc-136040), or by assessing the loss in PLC β 1 activity. PLC β 1 activity was assessed by measuring the ability of 20–50 nM enzyme to hydrolyze [³H]PI(4,5)P₂ dispersed on 2 mM sonicated phosphatidylserine:phosphatidylethanolamine: PI(4,5)P₂ (2:1:0.5, v/v) membranes as

described previously [10]. Trypsin digestion was carried out by adding 0.17 $\mu\text{g/ml}$ (final concentration) to PLC β 1 alone or incubated with a stoichiometric amount of α -synuclein at 25°C for specific times as noted in the text. μ -Calpain digestion was carried out similarly by adding μ -calpain in 800 μM calcium to pre-incubated PLC β 1- α -synuclein complexes.

Calpain digestion studies in SK-N-SH cells either untreated or transfected with siRNA(α -synuclein) (Dharmacon, Inc.), were carried out by increasing intracellular calcium by adding 1 μM of the calcium ionophore A23187 to the cell media DMEM containing 1.05 mM calcium. Cells were incubated at 37°C, 5% CO₂ for 0, 1, 2 and 4 hours. Control studies added 10 μM calpain inhibitor into the medium prior to addition of A23187, or did not contain A23187.

2.4 Pull down study to assess ubiquitination

SK-N-SH wild type cells and α -synuclein knock down cells were harvested and resuspended in lysis buffer containing 150mM NaCl, 2mM MgCl₂, 20mM Hepes (pH7.4) with 5mM 2-mercaptoethanol, 10 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ aprotinin and 1mM PMSF. After homogenization, cells lysates were spun down at 10,000 $\times g$, 4°C to remove cell debris and unbroken nuclei. Streptavidin beads were washed twice with PBS. 20 μl streptavidin beads were added to the cell lysate and the mixture was rotated for 1 hour at 4°C. The resin was spun down gently and the supernatant removed. The resin was then washed twice with 50mM Tris pH 8.0, 150mM KCl, 2mM EDTA, 5mM 2-mercaptoethanol, 1mM PMSF. PLC β 1 was eluted by adding buffer containing 2mM biotin and incubated on ice for 30min. The pulled-down proteins were assayed by western blotting using anti-PLC β 1 and anti-ubiquitin antibodies.

2.5 Mass spectrometry

PLC β 2 bands alone or subjected to calpain digestion were isolated on SDS PAGE electrophoresis. The bands were cut and removed, digested by trypsin and the peptides were analyzed by LC/MS/MS on a Thermo LTQ XL at the Proteomics Center at Stony Brook University.

2.6 RNA extraction, cDNA synthesis and real-time PCR analysis

Total RNA extraction, cDNA synthesis and real-time PCR were performed as described previously [11]. Briefly, control HEK cells and HEK cells overexpressing α -synuclein were rinsed twice in PBS prior to lysis in RLT buffer containing 1% β -mercaptoethanol (Qiagen, Valencia, CA). Total RNA was extracted from the cell lysate using the RNeasy Maxi Kit (Qiagen, Valencia, CA) with DNase I treatment to eliminate genomic DNA contamination. The RNA was quantitated by spectrophotometry, the samples were diluted to nominally identical concentrations and then quantitated a second time. First strand cDNA was synthesized from 5 μg total RNA using the Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). The cDNA samples were diluted 1:16 and real-time PCR was performed using the QuantiTect SyBr Green PCR kit (Qiagen, Valencia, CA) in an ABI 7300 Real Time PCR System (Applied Biosystems,). Each experimental sample was analyzed in triplicates. Threshold crossing points were converted into expression values automatically using the method by Larionov et al. [12]. Three primer pairs were used to amplify three different regions of PLC β 1, all within the common region between the two PLC β 1 isoforms previously reported [13]. The primer pairs were validated for equal efficiency using rat brain tissue as a positive control: (1) forward: 5' GAAGCAAGAGCAGGTCCAAG 3', reverse: 5' ACACAGCGACATCCAGACAG 3'; (2) forward: 5' CGGCCAGGCTATCACTACAT 3', reverse: 5' ATTCACCCATTCTCTGCTG 3'; (3) forward: 5' GGCTGGGAAGTCTGTCTGTAG 3', reverse: 5' TAAGGAGGGCATCACCAAAG 3'. PLC β 1 gene expression in each sample was normalized using 18S and 28S as internal

controls. The results from each primer pair were averaged. The specificity of amplification was verified by sequencing the amplicons from each sample.

3. RESULTS

3.1 The presence of α -synuclein increases the protein level of PLC β 1 in cultured cells

We observed that when α -synuclein is over-expressed in PC12 cells, the cellular level of PLC β 1 is greatly enhanced (see Fig. 1A). This effect is highly reproducible and occurs in a human neuroblastoma cell line SK-N-SH, in which both PLC β 1 and α -synuclein are endogenously expressed and in HEK293 cells that express low levels of PLC β 1 and no detectable α -synuclein (Fig. 1B–C). Additionally, down-regulation of α -synuclein by siRNA treatment lowers the cellular amount of PLC β 1 (Fig. 1C). In contrast, over-expression of PLC β 1 did not affect the expression levels of α -synuclein (Fig. 1C). These results show that α -synuclein may increase the cellular level of PLC β 1 in several cell types.

To determine whether α -synuclein is regulating the transcription of PLC β 1, we measured the message level of PLC β 1 in HEK293 cells that were transfected with α -synuclein or empty vector, using real-time PCR. The assay used three different PLC β 1 primer pairs to amplify distinct sites of the molecule (see Methods). All pairs gave identical results: expression of α -synuclein did not significantly change the transcript level of PLC β 1 (normalized values were 1.5 ± 0.4 empty vector versus 1.0 ± 0.4 α -synuclein, $n=3$). Thus, the increase in expression levels of PLC β 1 that occurs in the presence of α -synuclein is due to a post-transcriptional mechanism.

3.2 α -Synuclein prevents PLC β 1 degradation by proteases

The above results suggest that the increase of PLC β 1 with α -synuclein occurs at the protein level. We first tested whether the presence of α -synuclein alters PLC β 1 degradation through the ubiquitination pathway. We monitored the amount of ubiquitin-PLC β 1 with when α -synuclein levels are reduced using siRNA(α -synuclein) in SK-N-SH cells or when α -synuclein is over-expressed in HEK293 cells. The results, shown in Figure 2, suggest that the ubiquitination of PLC β 1 is not greatly affected by α -synuclein.

Another mechanism through which α -synuclein could increase the protein level of PLC β 1 is by protecting the enzyme from protease digestion. For these studies, we used purified protein expressed bacterially, dialyzed extensively and stored in 10% glycerol at -80°C . It has been reported that detergent-purified α -synuclein is an unfolded monomer, while native α -synuclein is a folded tetramer [14]. By native gel electrophoresis, we find that our preparation is approximately half monomer and half tetramer, and that its circular dichroism spectrum is identical to the one reported by Bartels and coworkers [14] (*data not shown*). We are presently determining whether α -synuclein associates to PLC β 1 as a monomer or tetramer.

We carried out an initial series of studies in which we compared the digestion of PLC β 1 and a mixture of PLC β 1 and α -synuclein (1:10) by trypsin. Trypsin cleaves peptide bonds of amino acids with positively charged side chains. We monitored digestion by both changes in activity, through ^3H -PI(4,5) P_2 hydrolysis, and western blotting of PLC β 1 (the amount of enzyme used was too low to be detected by coomassie staining). Digestion was followed for 1–5 minutes and was quenched by the addition of excess antitrypsin inhibitor. The results (Fig. 3) show a clear protection of PLC β 1 digestion by α -synuclein.

Trypsin digestion of proteins occurs in lysosomes and it is not clear whether protection of PLC β 1 by α -synuclein would give rise to the increased cellular levels seen in Figure 1. However, it has been found that cellular PLC β 1 is cleaved by the calcium-sensitive

protease, calpain resulting in 80 and 43 kDa protein products [15]. Calpain is a non-lysozymal protease that targets Cys residues contained in particular tertiary structural elements rather than sequence, and calpain has been shown to cleave a site in the C-terminus of the PLC β 1 (see [16]). We repeated the digestion study using μ -calpain and found that α -synuclein provides moderate but significant protection of PLC β 1 from calpain-mediated proteolysis (Fig. 4A). Interestingly, calpain treatment produced a more active enzyme (Fig. 4B); an effect that does not occur in the presence of α -synuclein.

To determine whether protection of calpain cleavage was general to other PLC β enzymes, we repeated this study using PLC β 2. We found that both α -synuclein and another family member γ -synuclein, efficiently protected PLC β 2 from calpain digestion (Fig. 4C). Like PLC β 1, calpain cleavage produced an active product but calpain did not digest a PLC β 2 mutant protein missing ~400 C-terminal residues (*data not shown*). Taken together, these results suggest that *in vitro*, α -synuclein protects PLC β 1–2 from calpain cleavage of the C-terminus.

3.3 Identification of the calpain cleavage site of PLC β 1

We carried out studies to determine the PLC β cleavage site protected by α -synuclein. Previously, Rhee and coworkers used a series of monoclonal antibodies to find that calpain cleaves PLC β 1 at or close to residue 880 [22]. We extended this previous studies to identify the calpain cleavage site of PLC β 2 using mass spectrometry. For these studies, we analyzed the intact protein and the 80 kDa degradation product. We find that the degradation product is missing the residues after 743. Thus, the calpain cleavage site of PLC β 2 is ~140 residues upstream from the site of PLC β 1 and lies at the end of the C2 domain and beginning of the C-terminal region of the enzyme. This result is consistent with our observation that calpain does not cleave the C-terminal deleted enzyme. We note that this 80 kDa fragment was not detected in cells by our commercial antibodies.

3.4 α -Synuclein protects PLC β 1 from calpain degradation in cultured cells

The above studies suggest that α -synuclein may protect PLC β 1 from calpain digestion in cells. We tested this idea in SK-N-SH cells. Activation of μ -calpain was induced by incubating the cells in a high calcium medium and by adding a calcium ionophore. Control studies were carried out in the presence of a calpain inhibitor (*see Methods*). A typical result is shown in Fig. 5A. The persistent appearance of PLC β 1 at longer digestion times suggests that calpain digestion is not efficient.

We then tested whether α -synuclein can protect PLC β 1 from calpain degradation. Since SK-N-SH cells express both endogenous α -synuclein and PLC β 1, we modulated the amount of α -synuclein by down-regulating its expression through the use of siRNA. These cells showed little degradation of PLC β 1 after addition of calcium ionophore. However, when the concentration of α -synuclein was reduced, PLC β 1 was strongly degraded (Fig. 5B). Although other mechanisms besides calpain activity may underlie these results, the simplest explanation is that α -synuclein protects PLC β 1 from degradation at high intracellular calcium levels.

4. DISCUSSION

In this study, we have shown that α -synuclein can increase the cellular level of PLC β through protection from degradation from enzymes such as μ -calpain. α -Synuclein has been the subject of many studies, however, its cellular function remains unclear. In cells, α -synuclein aggregation appears to be promoted by lipids and accumulation of ApoE, A β and components of the ubiquitin system (see [17],[4]). Interestingly, α -synuclein can reverse pathogenesis caused by deletion of a pre-synaptic chaperone protein [18]. Taken together,

these studies suggest a link between α -synuclein function, lipid interactions and chaperone activity.

Several years ago, we found that α -synuclein can affect the signaling properties of PLC β enzymes [6]. Since α -synuclein is highly expressed in neuronal lines, we focused this study mainly on PLC β 1 which has highest expression in these cell types [19]. We found that the presence of α -synuclein increases the level of PLC β 1 and that this stabilization does not appear to be due to changes in transcription or in ubiquitin-mediated degradation. Rather, we find that α -synuclein may be protecting PLC β 1 from degradation by proteases. While α -synuclein offers some protection from trypsin digestion, it is unlikely that this process contributes to the observed increase in PLC β 1 in the presence of α -synuclein since digestion by trypsin is lysosomes. However, proteolysis of PLC β 1 by calpain is very likely to be physiologically relevant, since it occurs in the cytosol and is calcium activated. Although regulation of PLC β 1 by calpain in neuronal cells has not yet been explored, calpain cleavage of PLC β 3 in human platelets appears to play an important role in platelet aggregation [20].

PLC β enzymes are composed of a catalytic domain flanked by several regulatory domains that mediate G protein and membrane association (see [21]). These domains, along with a “lid” that can occlude the active catalytic site, appear inhibitory [22]. Therefore, it is not surprising that removal of the C-terminus by calpain results in a significant increase in enzyme activity. Assuming this increase in PLC β activity results in a more pronounced release of intracellular Ca^{2+} that, in turn, promotes activation of μ -calpain, then this process can be thought of as a positive feedback loop.

Of the many members of the calpain family, two have been well-characterized: μ -calpain, which is activated at micromolar calcium levels or m-calpain, which is activated at millimolar calcium levels *in vitro* (for review see [16]). Although calpain targets Cys residues, it appears that specificity is dictated by structural elements as opposed to sequence. We find that calpain cleavage of rat PLC β 2 occurs at the end of its C2 domain at residue 753 with high efficiency. This site can be compared to that of human PLC β 1, for which cleavage by calpain has been reported to occur at residue 880 in the C-terminal domain [15]. The observation that calpain cleaves a Cys residue in the C2 domain of PLC β 2 suggests that it may target Cys residues in other C2 domains, especially in proteins involved in cytoskeletal assembly and disassembly, but this does not seem to be the case. While the higher efficiency of PLC β 2 cleavage by calpain *in vitro* is most likely due to the strong site at residue 753 as compared to PLC β 1, the lower efficiency in cells might result from endogenous α -synuclein, or factors that occlude the calpain site, such as basal interaction with $\text{G}\alpha_q(\text{GDP})$ [23].

Activation of PLC β 1 by $\text{G}\alpha_q$ results in release of intracellular calcium and opening of calcium channels to raise intracellular calcium and promote activation of μ -calpain. Calpain cleavage of PLC β 1–3 results in enzymes that are detached from $\text{G}\alpha_q$ mediated signals such as those triggered by epinephrine, serotonin and dopamine, thereby diminishing the potential for sustained calcium mobilization. The protection of PLC β enzymes from calpain cleavage by α -synuclein in SK-N-SH cells show that α -synuclein may prevent uncoupling of PLC β from G protein regulation and may also affect the cellular localization of the enzyme since $\text{G}\alpha_q$ contacts are lost. This protection may play a prominent role in neuronal cells where both α -synuclein and PLC β 1 are expressed at high levels. It is notable that calpain-degraded PLC β 2–3 can still be activated by $\text{G}\beta\gamma$ subunits release by stimulation of any $\text{G}\alpha$ family (see [24]).

Phage-display studies suggest that α -synuclein has many potential binding partners in cells, and we have found that PLC β 1–3 bind very strongly to this protein [6]. It is quite likely that α -synuclein has other cellular binding partners and it may similarly influence their

regulation. While PLC β 1 and other α -synuclein binding partners are expected to stabilize α -synuclein structure, it is possible that their loss or their aggregation promotes α -synuclein aggregation and subsequent pathogenesis. In summary, by modulating the cellular levels of specific proteins, α -synuclein might promote particular signaling pathways and cellular events.

Abbreviations

PLCβ	phospholipase C β
FRET	Forster resonance energy transfer

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Figure 1. α -Synuclein increases the cellular level of PLCβ1

A- Western blot showing the change in the level of PLCβ1 with over-expression of α -synuclein in PC12 cells. Rat aortic smooth muscle cells (A10), which do not express either PLCβ1 or α -synuclein were used as a control. Band intensities normalized to tubulin and averaged for 3 data sets are shown on the right. **B-** The same study as in **A** showing a reduced, but still significant effect in non-neuronal HEK293 cells. **C-** Studies in the neuronal cell line SK-H-SN. *Left* - Lanes 1–2 show that the level of α -synuclein is not affected by over-expression of PLCβ1 ($n=3$). Lanes 3–4 show an increase in PLCβ1 when α -synuclein was expressed at a level that was 29% over endogenous as determined by band density and correcting for β -actin levels. Lanes 5–6 show a reduction in the level of PLCβ1 when cells are treated with si(RNA) α -synuclein. *Right* – Control study showing changes in protein levels using a non-targetting siRNA (siRNA for GAPDH). siRNA(GAPDH) did not result in any changes in α -synuclein or PLCβ1 levels relative to actin.

Figure 2. α -Synuclein does not affect the ubiquitination state of PLC β 1

A - Endogenous PLC β 1 in SK-N-SH cells untreated and treated with si(RNA)- α -synuclein was immuno-precipitated and probed for ubiquitin using a monoclonal antibody (n=2). **B** - Change in PLC β 1 ubiquitination with α -synuclein over-expression in HEK293 cells where the ubiquitin band shown corresponds to the PLC β 1 band identified by western blotting, stripping and reprobing. Note that in both **A** and **B** the lanes were loaded with similar amounts of PLC β 1 so that the degree of ubiquitination can be more easily compared.

Fig. 3. α -Synuclein protects PLC β 1 from trypsin digestion

Top – Trypsin digestion (0.16 mg/ml final) of PLC β 1 at 37°C alone (lanes 1, 2) or with a 1:1 molar amount of α -synuclein. Bottom – loss of PLC β 1 activity, as seen by ^3H -IP $_3$ production by trypsin alone or at a 1:1 molar amount of α -synuclein.

Fig. 4. Calpain cleavage of PLC β 1 produces a fragment that is protected by α -synuclein
A – Protection of calpain digestion of PLC β 1 by α -synuclein as seen by western blotting. The bar graph underneath correspond to the band intensities normalized to actin. **B** – A parallel study showing the change in PLC β 1 activity, as seen by ^3H -PI production due to calpain alone and in the presence of a 4 fold excess of α -synuclein (*left*) and PLC β 2 by γ -synuclein (*right*). **C** – Protection from calpain digestion of PLC β 2 by α -synuclein and γ -synuclein as visualized by western blotting and silver staining. The red arrow indicates the major band resulting from calpain digestion that is absent in the presence of α -synuclein. Calpain cleavage also produced a more active enzyme as seen by ^3H -IP $_3$ production (*right*).

5A

5B

Fig. 5. α -Synuclein stabilization PLC β 1 in SK-N-SH cells

A – Calpain was activated by treating the cells with 1 μ M of the Ca^{2+} ionophore A23187 to allow Ca^{2+} entry into the cell and the amount of PLC β 1 degradation was probed in the absence and presence of calpain inhibitor was viewed by western blotting. The graph shows the ratio of band intensity ratio of PLC β 1 to actin. **B** – Degradation of PLC β 1 with calpain activation in control cells and one were α -synuclein was down-regulated using siRNA.