Expression of p25, an Aberrant Cyclin-Dependent Kinase 5 Activator, Stimulates Basal Secretion in PC12 Cells

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Although alterations in the functions of neurotransmitter systems have been implicated in the pathology of Alzheimer’s disease (AD), the mechanisms that give rise to these alterations are not well understood. The amount of p25, an aberrant cleavage product of p35 that activates cyclin-dependent kinase 5 (Cdk5), is elevated in AD brains. The role of Cdk5 in neurotransmitter release has been well established. In this study, we examined whether p25 was linked to altered neurotransmitter release in AD. Transient or stable expression of p25 significantly increased basal secretion of human growth hormone (hGH) or neurotransmitter in PC12 cells. Expression of a p25 phosphorylation-deficient mutant, T138A, inhibited basal hGH secretion relative to the p25 wild type, suggesting the involvement of Thr138 phosphorylation in secretion. The expression and activity of β-site amyloid precursor protein cleaving enzyme 1 (BACE1), a key protease in the generation of β-amyloid, are increased in AD brains. Our previous studies indicated that overexpression of BACE1 enhanced basal secretion of hGH in PC12 cells. Transient co-expression of p25 and BACE1 further stimulated spontaneous basal secretion. These results indicate a novel role for p25 in the secretory pathway and suggest that elevated levels of p25 and BACE1 in AD brains may contribute to altered neurotransmitter pathology of AD through enhancing spontaneous basal secretion.

INTRODUCTION

Alzheimer’s disease (AD), an irreversible, age-related, progressive brain disorder characterized by dementia, is prevalent among individuals over the age of 65. The brains of people with AD exhibit two pathological hallmarks: amyloid plaques and neurofibrillary tangles, which are insoluble deposits of β-amyloid (Aβ) and hyperphosphorylated tau protein, respectively. AD is also characterized by alterations in the function of the neurotransmitter systems that release acetylcholine, glutamate, norepinephrine, serotonin, and other chemical messengers among individuals over the age of 65. The brains of people with AD exhibit two pathological hallmarks: amyloid plaques and neurofibrillary tangles, which are insoluble deposits of β-amyloid (Aβ) and hyperphosphorylated tau protein, respectively. AD is also characterized by alterations in the function of the neurotransmitter systems that release acetylcholine, glutamate, norepinephrine, serotonin, and other chemical messengers (Lanari et al., 2006; Selkoe, 2002). Pharmacological intervention targeting these neurotransmitter systems forms the basis of current AD treatments. The concentration of norepinephrine and glutamate in cerebrospinal fluid (CSF), which is likely an estimate of neurotransmitter concentrations at synapses, are significantly higher in patients with AD than in control subjects (Csemansky et al., 1996; Elrod et al., 1997; Jimenez-Jimenez et al., 1998; Smith et al., 1985) although conflicting results have been reported (Jimenez-Jimenez et al., 1998). Together, these findings suggest that altered neurotransmitter release may be responsible for the pathogenesis of AD.

p25 is an aberrant cleavage product of p35, a protein that activates cyclin-dependent kinase 5 (Cdk5); further, p25/Cdk5 has been suggested to be involved in the formation of neurofibrillary tangles and Aβ in AD (Cruz et al., 2003; 2006). p25 expression and Cdk5 activity increase in sporadic AD brains (Patrick et al., 1999; Swatton et al., 2004; Tseng et al., 2002), although the increase in p25 is controversial (Tandon et al., 2003). Accumulating evidence indicates that Cdk5 controls neurotransmitter release through phosphorylation of the various substrates such as Munc18-1 and the P/Q-type Ca²⁺ channel (Barclay et al., 2004; Chung, 2008; Tomizawa et al., 2002). Considering the key role of Cdk5 in neurotransmitter release, we hypothesized that disturbances in the activity of Cdk5 due to p25 contributed to abnormal regulation of neurotransmitter release in presynaptic terminals.

In this study, we investigated the role of p25 in exocytosis in PC12 cells by using labeled norepinephrine or human growth hormone (hGH). The hGH system has been widely used to study the effects of co-transfected proteins on regulated secretion in secretory cells such as PC12 and bovine chromaffin cells (Chung et al., 1999; Lee et al., 2007; Sugita et al., 1999). The Ca²⁺-dependent secretion of dense core vesicles in PC12 cells is similar to synaptic vesicle exocytosis in neurons, making PC12 cells a practical model system in which to analyze regulated exocytosis in neurons (Choi et al., 2007; Sugita et al., 1999). Here, we show that the transient or stable expression of p25 significantly increases spontaneous basal secretion in PC12 cells.

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MATERIALS AND METHODS

Plasmids
Complementary DNAs (cDNAs) encoding human p25, Cdk5, and BACE1 were cloned from a human brain cDNA library by using the reverse transcription-polymerase chain reaction (RT-PCR) method, and were then subcloned into the pcDNA-myc-his expression vector (Invitrogen, USA). cDNA encoding the phosphorylation-defective p25T138A mutant (Thr138 → Ala138 as per the numbering for p35) was generated by Oplt-mediated site-directed mutagenesis; the sequences of the resulting clones were then verified.

Cell culture, transfection, and secretion assay
The PC12 cell preparation, transient transfection, and hGH secretion assay were performed as previously described (Lee et al., 2007). For hGH secretion experiments, PC12 cells in 12-well plates were transfected with plasmids encoding hGH and the protein of interest using Lipofectamine 2000 (Invitrogen). Two days after transfection, hGH secretion was measured by using an enzyme-linked immunosorbent assay (ELISA) kit (Roche, Switzerland). To measure constitutive secretion, we determined the amount of hGH secreted into the culture media during 2 days of incubation (normalized to the amount of hGH retained in the transfected cells). In the secretion experiments, transfected cells were incubated in a control physiological salt solution [PSS; 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, 15 mM HEPES (pH 7.4), 0.5 mM ascorbate, and 0.5% bovine serum albumin (BSA)] or in a depolarizing PSS buffer containing 100 mM KCl and 50.6 mM NaCl, and the amount of hGH secreted into the buffer and retained in cells at different time points was measured. The amount of secreted hGH was expressed as the percentage of total hGH.

Neurotransmitter uptake and release assay
The neurotransmitter uptake and release assay was performed as previously described (Chung et al., 1998; Lee et al., 2007). PC12 cells were plated on 48-well plates at 3 × 10^4 cells/ml. PC12 cells were pre-incubated for 3 h in culture medium containing 3[H]-norepinephrine [1-7.8[H]-norepinephrine, 37 MBq/ml, 1.0 mCi/ml] and 0.5 mM ascorbate. Cells were rinsed with control PSS buffer containing 0.5 mM ascorbate. Secretion was subsequently determined during 20 min incubation in PSS buffer containing 5.6 mM KCl or 100 mM KCl. The incubation solution was removed, and the cells were lysed with 1% Triton X-100. The radioactivity in the incubation solution and that in the cell were determined by liquid scintillation counting. Unless otherwise stated, data are represented as means ± SEM, with three samples per group.

PC12 Tet-Off cells stably expressing p25
PC12 Tet-Off cells (Clontech, USA) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS), 5% horse serum, and 100 μg/ml of G418, in 5% CO₂ at 37°C. To construct a cell line expressing p25 under the regulation of the Tet-Off system, p25 cDNA was subcloned into the pTRE2puro vector (Clontech) to obtain pTRE2puro-p25 cDNA. PC12 Tet-Off cells were transfected with pTRE2puro-p25 cDNA using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. At 48 h after transfection, the medium was replaced with RPMI 1640 medium containing 100 μg/ml of G418, 1 μg/ml of doxycycline, and 3 μg/ml of puromycin, and the medium was replaced every 4 days. Dead cells were separated from living cells at 5-7 days. After several washings with RPMI 1640 medium, only living cells were cultured on a 150-mm dish and an isolated single cell colony was transferred serially onto 96-, 24-, 12-, and 6-well plates in order. For protein expression, the clone was further cultured in RPMI 1640 medium lacking doxycycline for 3 days. Stable cell lines were selected by determining p25 expression by Western blotting.

Western blot analysis and immunocytochemistry
For protein expression, PC12 cells transfected with the indicated plasmids or stable PC12 Tet-Off cells were lysed in radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, 0.5% deoxycholic acid] containing 1 mM phenylmethylsulphonyl fluoride (PMSF) and a protease inhibitor cocktail. Equal amounts of lysates were subjected to western blot analysis. Antibodies to p25/p35 (C19) and Cdk5 (J3) were obtained from Santa Cruz Biotechnology (USA). For immunocytochemistry, PC12 cells were fixed and permeabilized 2 days after transfection. Cells were incubated with primary antibodies [rabbit anti-p25/p35 antibody (Santa Cruz Biotechnology) or mouse anti-hGH antibody (Zymed, USA) at 1:200 dilutions] for 2 h, and then incubated with secondary antibodies (Cy3 anti-rabbit antibody and Oregon Green 488 anti-mouse antibody at 1:500 and 1:100 dilutions, respectively) for 1 h. They were then examined under a confocal laser scanning microscope (LSM 510; Carl Zeiss, Germany).

RESULTS

Transient expression of p25 increases basal secretion in PC12 cells
To determine the effect of p25 on hGH secretion, expression plasmids encoding human p25 and hGH (pXGH5) were co-transfected into PC12 cells. As a negative control, PC12 cells were also co-transfected with a control expression vector (pcDNAmyc-his) and pXGH5. Overexpression of p25 in PC12 cells resulted in enhanced basal secretion of hGH relative to that of control cells within the first 2 min of incubation in PSS, and this enhancement continued for the next 13 min (Fig. 1A). The effect was observed in 19 independent experiments, with an average basal secretion enhancement of 46% ± 7% (p < 0.0001 versus control) during incubation for 15 min (Fig. 1B). Expression of the transfected proteins was determined by double immunocytochemistry and immunoblotting performed using PC12 cells (Figs. 1C and 3B). Consistent with previous reports (Chung et al., 1999; Khvotchev et al., 2003; Lee et al., 2007; 2008), most transfected cells expressed both p25 and hGH. Immunofluorescent localization of hGH revealed an extensive, fine punctuate pattern throughout the cell and widely dispersed p25 staining throughout the cells (Fig. 1C). In the merged image, hGH and p25 were partially co-localized (Fig. 1C).

We then determined whether the increase in basal secretion was correlated with an increase in constitutive hGH secretion. Transient expression of p25 had no effect on the amounts of hGH secreted into the culture media during 2 days (normalized to the amount of hGH retained in the transfected cells; Fig. 1B, constitutive secretion). These results indicated that spontaneous basal secretion in the absence of a stimulus may arise from secretion from the regulated pathway as previously reported (Lee et al., 2007; 2008; Matsuuchi and Kelly, 1991; Varro et al., 1996). Furthermore, in 19 independent experiments, transient expression of p25 slightly inhibited stimulus-dependent secretion by 13% ± 2% relative to the control cells (p < 0.0001 versus control) during 15 min of incubation (Fig. 1B), supporting a role