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Research report

Secreted form of β-amyloid precursor protein activates protein kinase C and phospholipase Cγ1 in cultured embryonic rat neocortical cells

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Abstract

The secreted form of β-amyloid precursor protein (sAPP) has been reported to exert various biological activities in cultured neurons. The signal transduction mechanisms underlying these physiological functions of sAPP remain unclear. We now report that treatment of neural cells with the secreted form of APP695 (sAPP695) leads to dose- and time-dependent increase in phosphorylation of the endogenous substrates with a molecular mass of 80, 57 and 43 kDa. Pretreatment of cells with protein kinase C (PKC) inhibitor H-7 reduced phosphorylation of the 80- and 43-kDa proteins in a dose-dependent manner. The effect of sAPP695 on the phosphorylation is mimicked by phorbol 12-myristate-13-acetate (PMA). Downregulation of PKC by prolonged treatment of cells with PMA abolished sAPP695-enhanced phosphorylation of the 80- and 43-kDa proteins, indicating PKC is involved in the sAPP695-enhanced phosphorylation of these proteins in the cells. We also suggest that the 80- and 43-kDa proteins phosphorylated by sAPP695-stimulation are the major PKC substrates myristoylated alanine-rich C-kinase substrate and growth-associated protein-43. Furthermore, we demonstrate that tyrosine phosphorylation of phospholipase Cγ1 and formation of inositol 1,4,5-trisphosphate were increased by sAPP695-stimulation. These observations suggest that sAPP695 induces the activation of the signaling pathways through a stimulation of phosphoinositide–PKC cascade. © 1998 Elsevier Science B.V.

Keywords: β-Amyloid precursor protein; GAP-43; Inositol 1,4,5-trisphosphate; MARCKS; Phospholipase Cγ1; Protein kinase C; Signal transduction

1. Introduction

Extracellular deposition of β-amyloid peptide (β/A4) in the brain is a characteristic pathological feature of Alzheimer's disease [7,17]. β/A4 is derived from a larger protein referred to as β-amyloid precursor protein (APP) [13,31]. APP is a membrane-spanning glycoprotein expressed in most mammalian tissues, and its highest levels are found in the nervous system. Multiple APP isoforms produced by alternative splicing of the mRNA have been identified. The major isoforms in the brain are APP695, APP751 and APP770 [8,13,14,24,26,32]. APP normally undergoes proteolytic cleavage inside the β/A4-sequence, resulting in release of the secreted form of APP (sAPP) into the extracellular space [5,23,38]. sAPP has been reported to be involved in the regulation of cell survival [18], proliferation [28], and cell adhesion [3,20,29]. sAPP has been found to induce neurite outgrowth in neurons and PC12 cells [18,20,29]. We have also demonstrated that sAPP695 and sAPP770 promote neurite outgrowth in cultured embryonic rat neocortical neurons [22]. Several effects of sAPP on the cellular signaling pathway have been reported. sAPP stimulates the activity of mitogen-activated protein kinase (MAPK) in PC12 cells [10], activates cGMP-mediated signaling [1] and high conductance potassium channels [6] in primary hippocampal neurons.

In order to obtain further information on the signal transduction mechanisms by sAPP, we investigated the effect of sAPP695 on the phosphorylation of the endogenous substrates by using cultured embryonic rat neocortical neural cells. Here, we report that sAPP695 activates protein kinase C (PKC) and phospholipase Cγ1 (PLCγ1), and stimulates inositol 1,4,5-trisphosphate (IP_3) formation. We also suggest that the endogenous substrates phosphorylated as a result of the stimulation with sAPP695 are myristoylated alanine-rich C-kinase substrate (MARCKS) and growth-associated protein-43 (GAP-43) which are major PKC substrates in neurons. These findings indicate that

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the PLC-PKC second messenger system is likely to be activated by sAPP695.

2. Materials and methods

2.1. Materials

Recombinant human sAPP695 was prepared by using the yeast expression system as described previously [22]. The sample from the yeast transfected with the empty vector was also prepared by the identical procedure [22]. [γ-^{32}P]ATP was purchased from Amersham (Buckinghamshire, UK). H-7 dihydrochloride (H-7) and Phorbol 12-myristate-13-acetate (PMA) were from RBI (Natick, MA). H-89 and KN-62 were from BIOMOL (Plymouth Meeting, PA). Anti-PLCγ1 polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Rat brain MARCKS [16], rat brain GAP-43 [30], rabbit polyclonal antibody raised against a synthetic peptide corresponding to rat brain MARCKS C-terminus and antibody raised against purified bovine brain GAP-43 were kindly supplied by Dr. H. Taniguchi (Fujita Health University, Aichi, Japan). Dulbecco's modified Eagle's medium (DMEM) was from Gibco (Grand Island, NY).

2.2. Cell culture and stimulation

Cerebral hemispheres were obtained from embryonic day 17 Wistar rats and mechanically dispersed and plated at a cell density of 1×10^7 cells per dish onto 35-mm tissue culture dishes coated with polyethylenimine. The neural cultures were maintained in serum-free DMEM at 37°C overnight in a 5% CO_2 humidified incubator. Under these conditions, neurons account for more than 95% of the culture population judging from the results of immunocytochemical staining with anti-neurofilament antibody [27]. The neural cultures were preincubated with DMEM for 10 min and then treated with the recombinant sAPP695 dissolved in the medium at 37°C. Cells were treated with the medium alone or the medium containing the same volume of the sample from the yeast transfected with the empty vector under the same conditions as a control.

2.3. Cell fractionation

After the treatment, cells were scraped and disrupted in 150 μl of ice-cold homogenizing buffer (20 mM Tris–HCl, pH 7.5, containing 2 mM EDTA; 0.5 mM EGTA and 0.25 M sucrose) with protease inhibitors and phosphatase inhibitor (0.1 mg of leupeptin per ml, 2 mM PMSF and 1 mM Na_3VO_4 on a sonicator by two 10-s bursts. The cell lysate was centrifuged at $500 \times g$ at 4°C for 10 min to remove the nuclei. The supernatant was then centrifuged at $150\,000 \times g$ at 4°C for 1 h to yield the cytosolic extract. The protein content was estimated with BCA Protein Assay Reagent (PIERCE, Rockford, IL).

2.4. In vitro protein kinase assay

The in vitro protein kinase assay was carried out by using the cytosolic extract of neural cells (containing 0.5 μg of proteins) and [γ-^{32}P]ATP (1 μM, 10 μCi) in 10 μl of kinase buffer (25 mM HEPES, pH 7.4, containing 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 2 mM dithiothreitol) at 30°C for 15 min. The reaction was terminated by the addition of 2.5 μl of 5 × Laemmli sample buffer, or by heating at 100°C for 5 min. The heat-treated mixture was centrifuged at $20\,000 \times g$ for 30 min to remove the denatured proteins, and the supernatant was mixed with 2.5 μl of 5 × Laemmli sample buffer. The phosphorylated proteins in the Laemmli sample buffer were subjected to SDS–PAGE with a 4–20% gradient gel (Daiichi Pure Chemicals, Tokyo, Japan) and visualized by autoradiography. For alkaline treatment, the gel was incubated with 1 M KOH at 55°C for 1 h, and washed with several changes of 25% methanol and 7% acetic acid. The gel was subsequently dried and the alkaline resistant proteins were visualized by autoradiography. To examine the effect of protein kinase inhibitors, the in vitro protein kinase assay was performed in the presence of either dimethyl sulfoxide (DMSO), used for dilution of H-89 and KN-62, or various concentrations of H-7 (in water), H-89 and KN-62 using the cytosolic extract of neural cells stimulated with 5 nM sAPP695 at 37°C for 10 min. Effect of H-7 in intact cells was examined by incubating cells with 30 or 60 μM H-7 for 1 h, and stimulated with the medium containing 5 nM sAPP695 followed by the in vitro protein kinase assay. For direct activation of PKC, the in vitro protein kinase assay was performed using the cytosolic extract of neural cells treated with the medium alone in the presence of 1 μM PMA. Downregulation of PKC was carried out by incubating neural cells with 250 nM PMA at 37°C for 18 h, and then stimulated with 5 nM sAPP695 for 10 min, followed by the in vitro protein kinase assay. Neural cells were preincubated with 0.005% DMSO and subsequently stimulated with either the medium alone or 5 nM sAPP695 under the same conditions as a control. In addition, all the cytosolic extract of cells used for the in vitro protein kinase assay contained 0.5 μg of proteins.

2.5. Detection of MARCKS and GAP-43

The in vitro protein kinase assay was carried out as described above by incubating the cytosolic extract of neural cells (containing 10 μg of proteins) with [γ-^{32}P]ATP (100 μM, 10 μCi) in 10 μl of kinase buffer at 30°C for 15 min. The mixture was separated on 4–20% SDS–PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) and visualized by autoradiography. The membrane was incubated with rabbit polyclonal antibodies to MARCKS and GAP-43 (diluted to 1:2000 and 1:1000, respectively), followed by anti-rabbit IgG conjugated to horseradish peroxidase (di-

luted to 1:3000, Amersham) and detection by the enhanced chemiluminescence system (Amersham).

In the immunoprecipitation experiment, the in vitro protein kinase assay was performed by incubating the cytosolic extract of sAPP695-stimulated cells (containing 50 μg of proteins) with [γ-^{32}P]ATP (1 μM, 10 μCi) at 30°C for 15 min, followed by incubation of the mixture with 4 μl of anti-MARCKS antibody at 4°C for 1 h. The immune-complex was recovered with 20 μl of 50% protein A/G PLUS-Agarose (Santa Cruz Biotechnology) and washed twice with lysis buffer (10 mM Tris–HCl, pH 7.5, containing 0.5 M NaCl, 0.5% NP-40 and 0.1 mM Na_3VO_4). Proteins were solubilized in 2 × Laemmli sample buffer, separated on 4–20% SDS–PAGE and visualized by autoradiography.

2.6. Immunoprecipitation and immunoblot analysis of PLCγ1

The cytosolic extract of neural cells (containing 380 μg of proteins) was preincubated with 20 μl of 50% protein A/G PLUS–Agarose at 4°C for 1 h, and the supernatant was incubated with 5 μg of anti-PLCγ1 polyclonal antibody or anti-phosphotyrosine monoclonal antibody (clone 4G10, Upstate Biotechnology, Lake Placid NY) at 4°C overnight. The immune-complex was collected with 30 μl of 50% protein A/G PLUS–Agarose, washed three times with lysis buffer and resuspended in 2 × Laemmli sample buffer. Proteins were separated on 2–15% SDS–PAGE and transferred to a PVDF membrane. The membrane was incubated with 4G10 anti-phosphotyrosine antibody or anti-PLCγ1 antibody (1 μg per ml), followed by anti-mouse IgG or anti-rabbit IgG conjugated to horseradish peroxidase and detection by the chemiluminescence method as described above.

2.7. IP_3 measurement

The neural cultures were preincubated with DMEM containing 10 mM LiCl at 37°C for 5 min and then exposed to 5 nM sAPP695 for the times indicated. The reaction was terminated by addition of a 0.2 volume of ice-cold 20% perchloric acid. The samples were kept on ice for 20 min and transferred to microtubes and then centrifuged at 2000 × g for 15 min at 4°C. The supernatants were neutralized by addition of ice-cold 1.53 M KOH for 20 min on ice. After centrifugation at 2000 × g for 10 min, aliquots of the supernatants were used for IP_3 analysis using an IP_3 assay kit (Amersham).

3. Results

3.1. Protein phosphorylation in the cytosolic extract of sAPP695-stimulated neural cells

In order to detect the changes in phosphorylation of the endogenous proteins in the sAPP695-stimulated cells, the in vitro protein kinase assays were performed. Embryonic day 17 rats neocortical cells were stimulated with either the medium alone or the medium containing recombinant sAPP695, which was prepared from the yeast expression system [22]. After the stimulation, the cytosolic extract of neural cells was prepared and subjected to the in vitro protein kinase assay (Fig. 1). The results showed that sAPP695 induces intensive phosphorylation of the endogenous substrates with a molecular mass of about 80, 57 and 43 kDa in a dose-dependent manner (Fig. 1A). The maximum level was obtained with 5 nM sAPP695 (Fig. 1A, lane 4). The cytosolic extract of neural cells treated with the medium alone did not show significant phosphorylation of these proteins (Fig. 1A, lane 1). Phosphorylation of the proteins was also dependent on the duration of cell incubation with sAPP695 (Fig. 1B). sAPP695-enhanced phosphorylation of the proteins reached its maximum level within 10 min (Fig. 1B, lane 4). On the other hand, treatment of cells for 10 min with the same volume of the sample from the yeast transfected with the empty vector did not induce significant phosphorylation of the endogenous substrates (Fig. 1B, lane 5).

To characterize the protein kinases involved in sAPP695-enhanced phosphorylation of the proteins, we first examined heat stability and alkaline sensitivity of these endogenous substrates after the in vitro protein kinase assay. The 80- and 43-kDa proteins were resistant to heat treatment, whereas the 57-kDa protein disappeared by this treatment (Fig. 2A, lane 3). When the same gel was

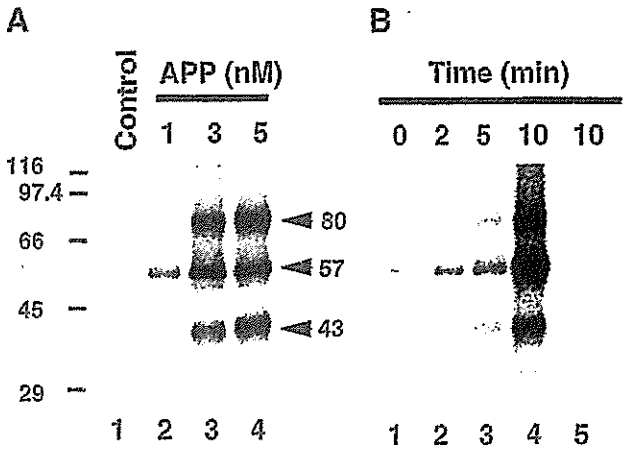

Fig. 1. Concentration dependence and time course of sAPP695-enhanced protein phosphorylation. (A) Neural cells were incubated with the medium alone (Control) (lane 1) or indicated concentrations of sAPP695 (APP) (lanes 2–4) for 10 min. The cytosolic extract of cells was subjected to the in vitro protein kinase assay as described in Section 2. The reaction was terminated by adding Laemmli sample buffer, and the phosphorylated proteins were separated on SDS–PAGE followed by autoradiography. The positions and sizes of the molecular mass markers and the phosphorylated proteins are shown on the left and right, respectively (sizes are in kDa). (B) Neural cells were incubated with 5 nM sAPP695 for the times indicated (lanes 1–4). Cells were also treated with the same volume of the sample prepared from the yeast transfected with the empty vector for 10 min (lane 5).

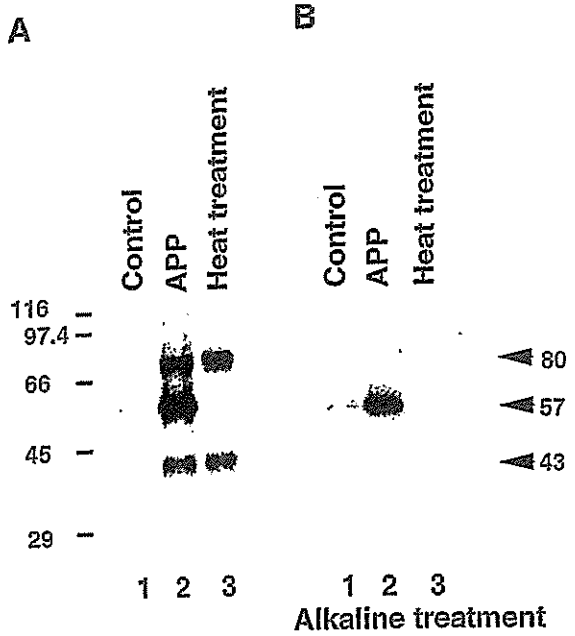

Fig. 2. Heat stability and alkaline sensitivity of the endogenous substrates. (A) Cells were treated with the medium or 5 nM sAPP695 for 10 min, and the cytosolic extract of cells was subjected to the in vitro protein kinase assay. The reaction was terminated by adding Laemmli sample buffer (lanes 1 and 2) or heating (lane 3) and the phosphorylated proteins were analyzed by SDS–PAGE followed by autoradiography. The positions and sizes of molecular mass markers are shown on the left (sizes are in kDa). (B) The alkaline treatment of the gel shown in (A). The positions of the phosphorylated proteins are shown on the right (sizes are in kDa).

subjected to alkaline treatment, the 80- and 43-kDa bands completely disappeared (Fig. 2B, lanes 2 and 3), but the 57-kDa band was still prominent (Fig. 2B, lanes 1 and 2). These results indicated that the 80- and 43-kDa proteins were phosphorylated at serine and/or threonine residues, and the 57-kDa protein was mostly phosphorylated at tyrosine residues.

3.2. *Effect of protein kinase inhibitors*

Next, we investigated the effect of various protein kinase inhibitors on the phosphorylation. The in vitro protein kinase assay was performed with the cytosolic extract of neural cells in the presence of either DMSO or various concentrations of protein kinase inhibitors. DMSO (a final concentration of less than 0.05%) used as a vehicle did not affect phosphorylation of the proteins (Fig. 3A, lanes 1 and 2). PKC inhibitor H-7, that inhibits PKC with an IC_{50} value of 6.0 μM [11], markedly reduced phosphorylation of the 80- and 43-kDa proteins in a dose-dependent manner (Fig. 3A, lanes 3–5). By contrast, phosphorylation of the 57-kDa protein was partially reduced by H-7. Since H-7 also inhibits cyclic AMP-dependent protein kinase with an IC_{50} value of 3.0 μM, we examined the effect of H-89, which specifically inhibits cyclic AMP-dependent protein kinase with an IC_{50} value of 0.05 μM [4]. H-89 did not significantly reduce phosphorylation of the proteins (Fig. 3A, lanes 6 and 7). We also examined the effect of KN-62, which specifically inhibits Ca^{2+}/Calmodulin kinase II with an IC_{50} value of 0.9 μM [34], and found that the phosphorylation was not significantly inhibited by 5 μM KN-62 (Fig. 3A, lane 8). These results suggest that PKC is mainly involved in sAPP695-enhanced phosphorylation of the 80- and 43-kDa proteins.

We further examined the effect of H-7 on sAPP695-enhanced phosphorylation of the proteins in intact cells (Fig. 3B). Pretreatment of cells with 60 μM H-7 for 1 h followed by activation with 5 nM sAPP695 completely inhibited phosphorylation of the 80- and 43-kDa proteins. On the other hand, phosphorylation of the 57-kDa protein was partially inhibited by this treatment (Fig. 3B, lane 3). The extent of the inhibition depends on the concentration of H-7, suggesting that H-7 inhibits PKC-mediated phosphorylation of the proteins in intact cells. These results taken together show that PKC is a major component in the kinase activities that stimulated by sAPP695 in neural cells. In addition to this, a partial inhibition of phosphorylation of the 57-kDa protein by H-7 may indicate that, besides protein tyrosine kinase, PKC is also involved in phosphorylation of this protein.

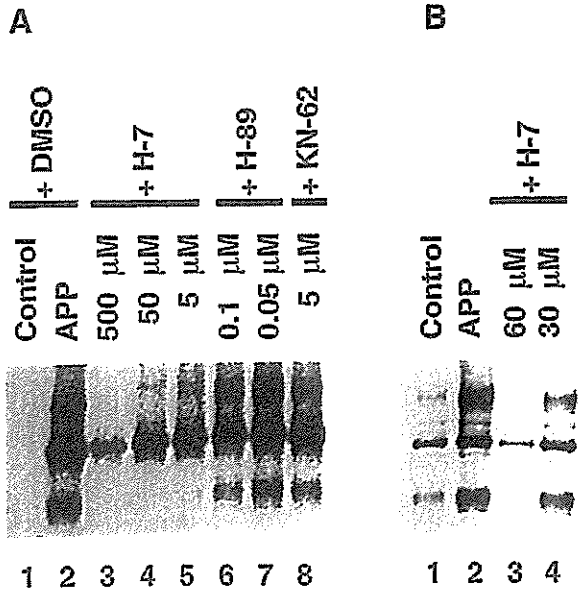

Fig. 3. Effect of protein kinase inhibitors on sAPP695-enhanced phosphorylation. (A) Cells were treated with the medium or 5 nM sAPP695 for 10 min. The in vitro protein kinase assay was performed with the cytosolic extract of cells in the presence of either DMSO (lanes 1 and 2) or various concentrations of protein kinase inhibitors (lanes 3–8). (B) Effect of H-7 on sAPP695-enhanced phosphorylation of the proteins in intact cells. Cells were preincubated with either vehicle (lanes 1 and 2) or H-7 (lanes 3 and 4) at 37°C for 1 h and then stimulated with the medium (lane 1) or 5 nM sAPP695 (lanes 2–4) for 10 min. The cytosolic extract of cells was subjected to the protein kinase assay and analyzed by SDS–PAGE followed by autoradiography.

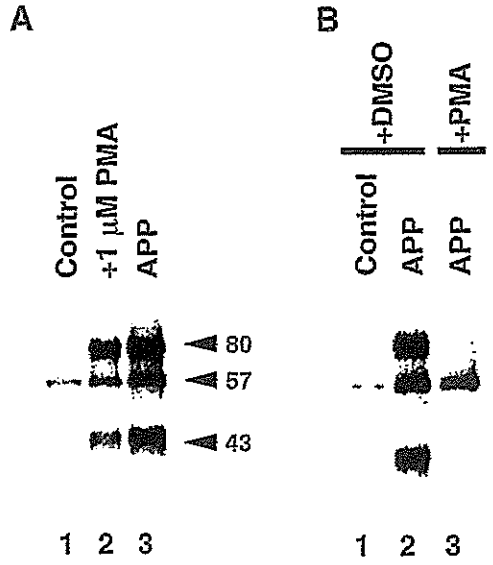

Fig. 4. Effect of PMA on phosphorylation of the endogenous substrates. (A) Direct activation of PKC in the in vitro protein kinase assay. Cells were treated with the medium (lanes 1 and 2) or 5 nM sAPP695 (lane 3) for 10 min, and the cytosolic extract of cells was subjected to the in vitro protein kinase assay with (lane 2) or without (lanes 1 and 3) 1 μM PMA. (B) Downregulation of PKC in cells by PMA. Cells were treated with 0.005% DMSO (lanes 1 and 2) or 250 nM PMA (lane 3) for 18 h and stimulated with the medium (lane 1) or 5 nM APP695 (lanes 2 and 3) for 10 min, followed by the in vitro protein kinase assay.

3.3. Effect of PMA

If sAPP695-enhanced phosphorylation of the proteins is due to the result of activation of PKC in the cells, direct activation of PKC in vitro should also induce the enhanced phosphorylation of the same endogenous substrates. Thus, we have activated PKC by PMA in the in vitro protein kinase assay and compared the phosphorylation pattern to that induced by sAPP695. The cytosolic extract of neural cells treated with the medium alone did not show significant phosphorylation of the proteins (Fig. 4A, lane 1), whereas addition of 1 μM PMA induced intensive phosphorylation of the 80- and 43-kDa proteins and slightly increased that of the 57-kDa protein (Fig. 4A, lane 2). Furthermore, we examined the effect of downregulation of PKC by prolonged treatment of cells with PMA on the phosphorylation of these proteins. The living cells were incubated with either 0.005% DMSO (Fig. 4B, lanes 1 and 2) or 250 nM PMA (Fig. 4B, lane 3) at 37°C for 18 h and then stimulated with the medium alone (Fig. 4B, lane 1) or the medium containing 5 nM sAPP695 (Fig. 4B, lanes 2 and 3) followed by the in vitro protein kinase assay. Pretreatment of neural cells with DMSO did not affect phosphorylation of the proteins. On the other hand, downregulation of PKC by prolonged PMA-treatment completely abolished sAPP695-enhanced phosphorylation of the 80- and 43-kDa proteins, and slightly decreased an intensity of the 57-kDa band (Fig. 4B, lane 3). These results strongly suggest that sAPP695 activates PKC in intact cells.

3.4. Identification of the 80- and 43-kDa proteins

Since our findings indicate that activation of PKC is induced by sAPP695, it is conceivable that the 80- and 43-kDa proteins phosphorylated by sAPP695-stimulation are the PKC substrates. Judging from their size, heat stability and sensitivities to alkaline treatment (Fig. 2), we

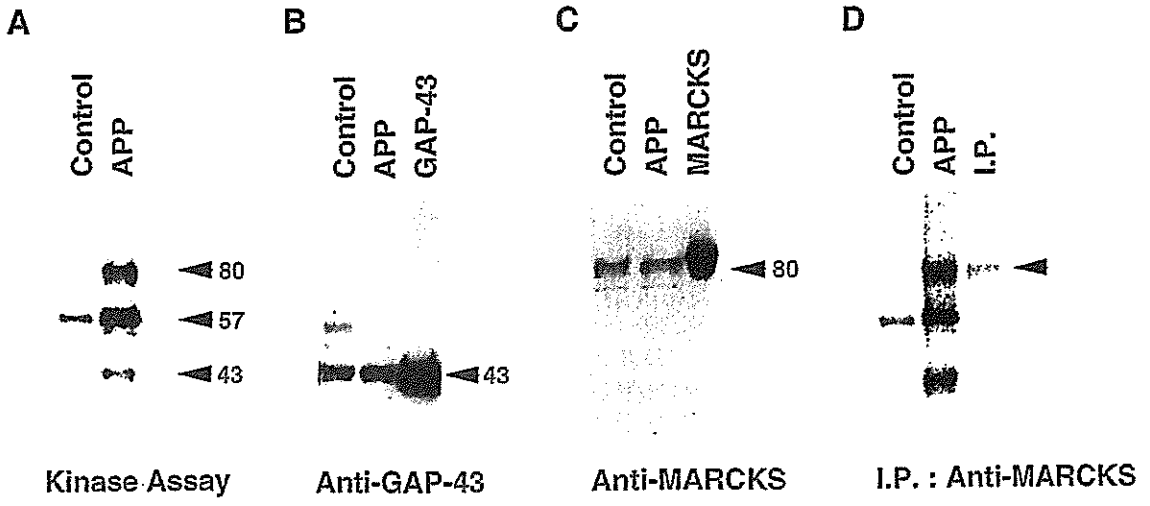

Fig. 5. Identification of the 80- and 43-kDa proteins. (A) The in vitro protein kinase assay was performed using the cytosolic extract of neural cells treated with the medium or 5 nM sAPP695 for 10 min. The phosphorylated proteins were separated on SDS–PAGE, transferred to a PVDF membrane and visualized by autoradiography. (B) The membrane was probed with anti-GAP-43 antibody. Rat brain GAP-43 was loaded as a positive control (GAP-43). (C) Immunoblot analysis with anti-MARCKS antibody. Rat brain MARCKS was loaded as a positive control (MARCKS). (D) Immunoprecipitation of MARCKS after the protein kinase assay. An autoradiogram of the protein kinase assay is shown on the left and middle lanes. Immunoprecipitation with anti-MARCKS antibody was performed on the cytosolic extract of sAPP695-stimulated cells after the protein kinase assay (I.P.). The position of MARCKS is shown by an arrowhead on the right.

suspected that the 80- and 43-kDa proteins are the major PKC substrates, MARCKS and GAP-43, respectively. To examine these possibility, we performed immunoblot analysis after the in vitro protein kinase assay. The phosphorylated proteins together with authentic MARCKS or GAP-43 were separated on SDS–PAGE, blotted to a PVDF membrane and visualized by autoradiography (Fig. 5A). The membrane was then immunoblotted with a polyclonal antibody raised against bovine brain GAP-43 (Fig. 5B) or rat brain MARCKS (Fig. 5C). The phosphorylated 80- and 43-kDa proteins migrated to the same positions as authentic MARCKS and GAP-43, and were immunoreactive with the respective antibodies. Moreover, when the sample of sAPP695-stimulated cells was incubated with anti-MARCKS antibody after the in vitro protein kinase assay, the 80-kDa protein was clearly immunoprecipitated (Fig. 5D).

3.5. sAPP695 increases tyrosine phosphorylation of PLCγ1

PKC is known to be activated by diacylglycerol (DAG) which is generated from phospholipase C (PLC)-mediated hydrolysis of inositol phospholipids. Our findings on activation of PKC by sAPP695 led us to speculate that sAPP695 also activates PLC. To investigate this speculation, we examined whether tyrosine phosphorylation of PLCγ1 was increased by sAPP695-stimulation, because PLCγ1 is predominantly expressed in the brain. The cytosolic extract of cells treated with either the medium alone or 5 nM sAPP695 were immunoprecipitated with anti-PLCγ1 antibody and subsequently immunoblotted with anti-phosphotyrosine antibody, 4G10 (Fig. 6A). The cytosolic extract of cells stimulated with 5 nM sAPP695 revealed the increase of tyrosine phosphorylation of several proteins in anti-PLCγ1 immunoprecipitates. The most prominent band with an apparent molecular mass of around 145 kDa was identified as PLCγ1 by reprobing the same membrane with anti-PLCγ1 antibody (Fig. 6B). As shown in Fig. 6B, similar amounts of PLCγ1 were present in the cytosolic extract of neural cells treated with the medium alone or sAPP695. Additional bands detected in the immunoprecipitates are likely to be the PLCγ1-associated proteins. To confirm whether tyrosine phosphorylation of PLCγ1 was indeed stimulated in the cytosolic extract of sAPP695-treated cells, the samples were immunoprecipitated with anti-phosphotyrosine antibody and subsequently immunoblotted with anti-PLCγ1 antibody (Fig. 6C). Again, tyrosine phosphorylation of PLCγ1 was significantly increased in the cytosolic extract of neural cells stimulated with sAPP695. These results suggest that the stimulation of cells with sAPP695 induces an activation of PLCγ1.

3.6. Effect of sAPP695 on cellular IP_3 formation

Activation of PLCγ1 has been known to generate IP_3 as a result of hydrolysis of inositol phospholipids. To confirm the activation of PLCγ1 induced by sAPP695, we examined the effects of sAPP695 on the formation of IP_3 in neural cells. Stimulation of cells with 5 nM sAPP695 caused a significant increase in IP_3 content in the cells

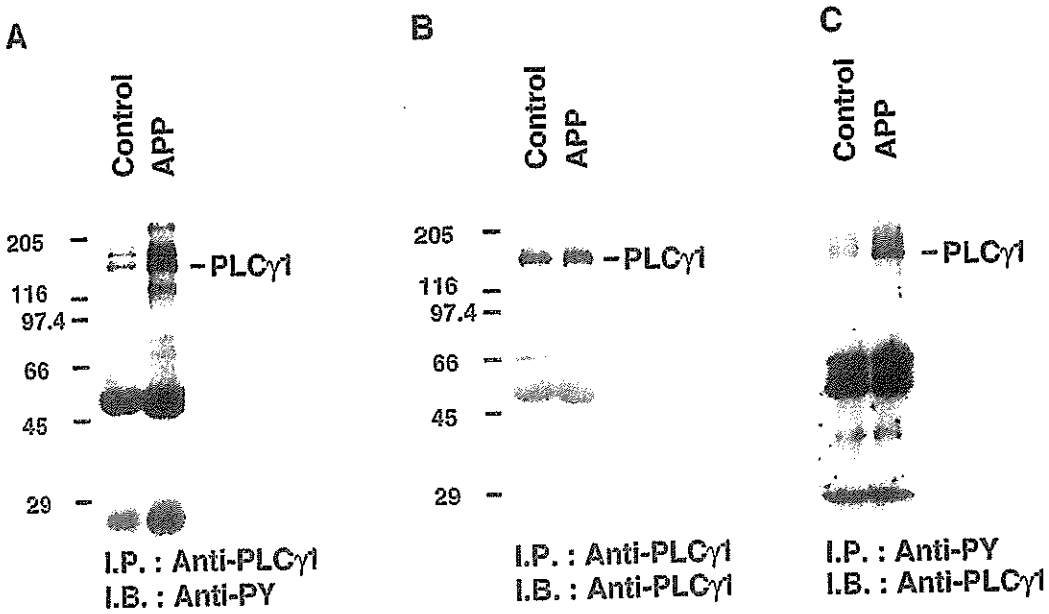

Fig. 6. Tyrosine phosphorylation of PLCγ1 by sAPP695. (A) The cytosolic extract of cells was immunoprecipitated (I.P.) with anti-PLCγ1 antibody and after SDS–PAGE and electrotransfer, the membrane was immunoblotted (I.B.) with anti-phosphotyrosine (PY) antibody. (B) The same membrane was reprobed with anti-PLCγ1 antibody. (C) The cytosolic extract of cells was immunoprecipitated with anti-phosphotyrosine antibody and subsequently immunoblotted with anti-PLCγ1 antibody. The data shown are representative of three similar experiments. The positions and sizes of the molecular mass markers are shown on the left (sizes are in kDa).

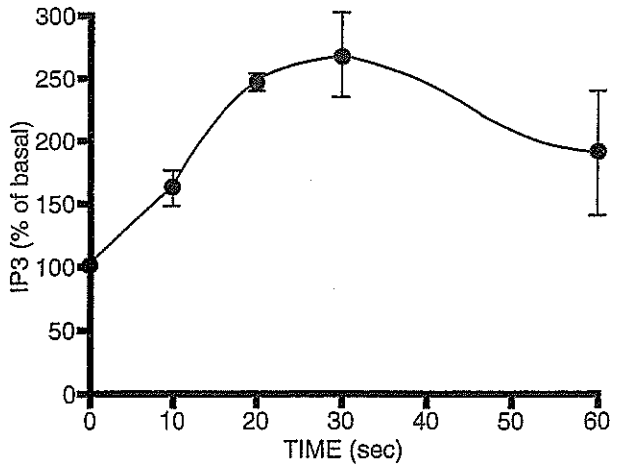

Fig. 7. Enhanced formation of IP_3 by sAPP695. Cells were treated with 5 nM sAPP695 for the times indicated. IP_3 content was measured as described in Section 2. Values are mean ± S.E. of three separate experiments.

peaked at 30 s (Fig. 7). The result indicated that sAPP695 stimulates phosphoinositide hydrolysis by PLCγ1 in neural cells.

4. Discussion

To detect the protein kinases that are specifically activated by sAPP695-stimulation, we first performed the in vitro protein kinase assay by using the cytosolic extract of neural cells. This experiment enabled us to detect sAPP695-enhanced phosphorylation of the endogenous substrates with a molecular mass of 80, 57 and 43 kDa.

We demonstrate that treatment of the kinase assay sample or cells with PKC inhibitor H-7 completely blocked the sAPP695-induced phosphorylation of the 80- and 43-kDa proteins and partially reduced phosphorylation of the 57-kDa protein (Fig. 3). Direct activation of PKC by application of PMA in vitro also induced intensive phosphorylation of the 80- and 43-kDa proteins and slightly increased phosphorylation of the 57-kDa protein (Fig. 4A). Moreover, downregulation of PKC in cells by prolonged treatment with PMA led to the loss of phosphorylation of the 80- and 43-kDa proteins (Fig. 4B). These observations strongly suggest that sAPP695-enhanced phosphorylation of the proteins is due to activation of PKC in intact cells. Additionally, the results of immunoblot and immunoprecipitation experiments suggest that the 80- and 43-kDa proteins are most likely to be the major PKC substrates, MARCKS and GAP-43, respectively (Fig. 5).

If sAPP695 induced the activation of PKC, it is reasonable to expect an activation of PLC which generates DAG, the physiological activator of PKC. In fact, we showed that treatment of cells with sAPP695 increased tyrosine phosphorylation of PLCγ1 (Fig. 6), indicating that sAPP695 activates PLCγ1 in intact cells. Furthermore, we demonstrate that the formation of IP_3 in neural cells was increased by sAPP695-stimulation (Fig. 7). These results strongly suggest that PLCγ1 and PKC are stimulated by sAPP695 in neural cells.

sAPP695 has been reported to reveal various biological activities in development of the brain. Among these functions of sAPP695, a number of studies have indicated that it plays an important role in the regulation of neurite outgrowth [19,20,29]. Our previous findings that sAPP695 promotes neurite outgrowth in cultured rat neocortical neurons [22] support these observations. Furthermore, our present results showing that sAPP695 stimulates PKC activity support these ideas, since activation of PKC has been shown to enhance neurite outgrowth in developing neurons and several cell lines [2,12,33]. In sympathetic neurons, conventional PKC, which shows Ca^{2+}-dependency for the activity [21], is localized to growth cones and activates neurite outgrowth [2]. In addition, intraneuronal delivery of a pseudosubstrate peptide of PKC, a specific inhibitor of conventional PKC, leads to collapse of growth cones in cultured embryonic rat cortical neurons [33]. In PC12 cells, overexpression of PKCε, a Ca^{2+}-independent isoform of PKC, enhances nerve growth factor-induced phosphorylation of MAPK and neurite outgrowth [12].

PKC has been reported to mediate MAPK activation through a poorly understood mechanism involving the activation of Ras [37] and Raf kinase [15,35]. Our present findings on activation of PKC by sAPP695 suggest the possibility that sAPP695 leads to activation of MAPK. In fact, this possibility is supported by an earlier report that sAPP stimulates the Ras-dependent MAPK cascade in PC12 cells [10]. Since MAPK has a wide variety of cellular targets, activation of MAPK by sAPP695 may have a number of functional effects on neural cells.

In this study, we observe potent phosphorylation of two endogenous PKC substrates, the 80- and 43-kDa proteins, after stimulating neural cells with sAPP695. On the other hand, according to the results of alkaline treatment, the 57-kDa protein was phosphorylated at tyrosine residues, indicating that protein tyrosine kinases are also involved in sAPP695-enhanced phosphorylation of the proteins. We suspect that the 57-kDa protein was a member of the Src family protein tyrosine kinase, because Src has been shown to be a PKC substrate [9,25] and the Src family consists of major cytosolic protein tyrosine kinases in various cells, including neurons [36]. The possible contribution of Src family tyrosine kinase to sAPP695-enhanced phosphorylation of the proteins remains to be determined. However, the present findings that tyrosine phosphorylation of PLCγ1 is also stimulated by sAPP695 suggest that certain tyrosine kinases are involved in the sAPP695-induced signal transduction. Our findings on activation of PLCγ1 and PKC by sAPP695 also suggest the possibility that sAPP695 stimulates phosphatidylinositol metabolism through interaction with its cell surface receptor-like molecule. Determination of the specific receptor-like

molecule for sAPP695 will provide further understanding of the molecular mechanisms involved in the biological activity of sAPP695.

Finally, in this study, we demonstrate that sAPP695 activates signaling molecules in neural cells. However, effects of other isoforms of sAPP, for example sAPP751 and sAPP770, remain unknown. The effects of these isoforms on the signaling cascade must be elucidated in future investigation.

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