Expression of syntaxin 1C, an alternative splice variant of HPC-1/syntaxin 1A, is enhanced by phorbol-ester stimulation in astroglioma: participation of the PKC signaling pathway[1]

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Abstract Syntaxin 1C is an alternative splice variant of HPC-1/syntaxin 1A; the latter participates in neurotransmitter release and is assigned to the gene domain responsible for Williams' syndrome (WS). It is expressed in the soluble fraction extracted from human astroglioma cell lines T98G and U87MG. Quantitative immunoblot and indirect immunofluorescence analyses revealed that the expression of syntaxin 1C was upregulated by phorbol 12-myristate 13-acetate (PMA), but not by forskolin. A protein kinase C (PKC) inhibitor suppressed this enhancement. These results suggest that syntaxin 1C expression is regulated via the PKC signal pathway. This is the first report of a signal transduction system that directly affects the expression of syntaxin protein.

Key words: Syntaxin 1; Astroglioma; Phorbol-ester; Protein kinase C; Protein kinase C inhibitor

1. Introduction

According to the soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptor (SNARE) hypothesis explaining the mechanism of intracellular transport, syntaxins function in intracellular membrane fusion events [1–4]. 18 mammalian syntaxins have been identified, most of which are associated with specific intracellular membrane compartments by virtue of their carboxyl-terminal hydrophobic transmembrane domain (TMD) [5,6]. Recently, several syntaxin isoforms lacking a TMD have been reported. Syntaxin 11 behaves like membrane protein, probably via lipid anchoring, despite the lack of an obvious traditional TMD [7,8]. Syntaxins 2D, 3D and 16C, which are generated by alternative splicing, are isoforms without a TMD [9–11]. However, the functions of syntaxins lacking a TMD have not been elucidated.

Syntaxin 1C, which lacks the hydrophobic carboxyl-terminal containing the TMD characteristic of the syntaxin family, is generated by alternative splicing of syntaxin 1A (HPC-1) [12]. The latter participates in neurotransmitter release [1,6]. The syntaxin 1A and 1C genes exist in the gene domain that causes the congenital neurodevelopmental disorder Williams' syndrome (WS) when deleted [13,14].

Recently, it was suggested that expression of syntaxin 1A and 1B mRNA was regulated in neuronal cells [15,16]. However, it is not known whether the expression of syntaxin protein isoforms lacking a TMD is also regulated.

This paper reports that syntaxin 1C is expressed in astroglial cells as a soluble protein. Furthermore, immunoblotting and indirect immunofluorescence analyses revealed that syntaxin 1C protein expression was enhanced by phorbol-ester stimulation, but not by forskolin or a non-effectable analog of phorbol-ester, and that protein kinase C (PKC) inhibitor treatment blocked the enhanced expression of syntaxin 1C protein caused by phorbol-ester.

2. Materials and methods

2.1. Reverse transcriptase-polymerase chain reaction (RT-PCR) and DNA cloning

Total cellular RNA was extracted using a QIA-Amp RNA extraction kit (Qiagen). RT-PCR analysis was carried out using an RNA PCR kit (TAKARA) according to the manufacturer's instructions. The primers used to distinguish human syntaxins 1A and 1C in the RT-PCR analysis were 5′-CTTTGCCTCTGGGATCATCATGG-3′ (syntaxin 1 sense) and 5′-CCTTGCTCTGGTACTTGACGGCC-3′ (syntaxin 1 antisense). For semi-quantitative PCR analysis [17], the primer for β-actin as an internal control was purchased from Maxim Biotech, Inc. The PCR cycling profile for syntaxin consisted of a 5-min initial denaturing at 95°C, followed by 40 cycles of 95.5°C for 1 min, 55°C for 0.5 min, and 72°C for 0.5 min using a Perkin-Elmer 9700 thermal cycler. The amplification products were stained with ethidium bromide. To clone human syntaxins, the following oligonucleotide primers were designed: 5′-TTAGATCTATGAAGGACCGAACCCAGGAG-3′ (syntaxins 1A and 1C sense), 5′-AAGAATTCTAGGCGAAGATGCCCCCAAC-3′ (syntaxin 1A antisense), and 5′-AAGAATTCTCAATCATCTCTCCCTGCGG-3′ (syntaxin 1C antisense). These primers were used in RT-PCR with mRNA isolated from a human brain library (BIO101) and T98G human astroglioma cells (see Fig. 1B). The full-length human syntaxin

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[1] The nucleotide sequences of human HPC-1/syntaxin 1A and syntaxin 1C cDNA have been deposited in the DDBJ nucleotide sequence database under accession numbers D37932 and AB086954M, respectively.

Abbreviations: SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptor; TMD, transmembrane domain; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; GF, GF109203X

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cDNAs were inserted into the *Bam*HI and *Eco*RI sites of pcDNA3 vector (Invitrogen). In addition, human syntaxins 1A and 1C used as controls for immunoblotting were amplified using the following primers: 5′-TTAGATCTATGAAGGACCGAACCCAGGAG-3′ (syntaxins 1A and 1C sense), 5′-AACTCGAGTAGGCGAAGATGCCCCC-AAC-3′ (syntaxin 1A antisense) and 5′-AACTCGAGTCAATCA-TCTCTCCCTGCGG-3′ (syntaxin 1C antisense). Syntaxins 1A and 1C were subcloned into the *Bam*HI and *Xho*I sites of pPROEX-1 vector (Life Technologies). The sequences of the subcloned syntaxins were confirmed using an ABI 377 sequencer (ABI). The recombinant proteins were purified as reported [18].

2.2. Preparation of anti-syntaxin 1C antiserum

To prepare antiserum against syntaxin 1C, a peptide (*pep-1C*: CPEPQPNPEEGALWSSGAPGPAGRDD) unique to human syntaxin 1C was synthesized with a peptide synthesizer. This peptide was purified by high performance liquid chromatography and confirmed by mass spectroscopic analysis. Antiserum against pep-1C peptide was raised in rabbit and IgG was purified as described [19].

2.3. Cell culture and drug treatment

T98G, NB-1, IMR-32, and PC12h cell lines were purchased from HSRRB (Japan). Human astroglioma cell lines (U87MG, U138, U343MGa, and SF126) were kindly provided by Dr. Hiroki Sawa (Kyorin University, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) (Sigma), penicillin (100 μg/ml), and streptomycin (100 μg/ml).

For drug stimulation, the cells were treated for 3–48 h with 1–10 μM phorbol 12-myristate 13-acetate (PMA) (Sigma), 10 μM 4α-PMA (Sigma), or 10 μM forskolin (RBI). For the PKC blocking experiment, cells were pretreated for 2 h with 5 μM GF109203X (GF), a bisindolylmaleimide, as a general PKC inhibitor (Calbiochem), and then incubated for 12 h both with PMA and GF.

2.4. Isolation of transformants overexpressing syntaxin

T98G and U87MG cells grown on 90-mm plastic dishes were trypsinized after washing with phosphate-buffered saline (PBS) twice, and resuspended into 0.3 ml of serum-free DMEM on ice. Using a Bio-Rad gene pulsar, 1×10^6 cells were electro-transfected with 150 μg/ml of pcDNA3 syntaxins. The electroporation was carried out at a field strength of 0.75 kV/cm and a capacitance of 960 μF. In order to obtain transformants, the cells were cultured in DMEM containing neomycin (800 μg/ml) for 2 weeks. Ultimately, three syntaxin transformant cell lines were isolated.

2.5. Subcellular fractionation

Cells grown in 75-cm^2 dishes were fractionated as described previously with slight modification [20]. The cells were scraped out with a rubber policeman into 0.5 ml of ice-cold homogenization buffer (250 mM sucrose, PBS, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml pepstatin, 10 μg/ml aprotinin, 3 μg/ml leupeptin, pH 7.4) and homogenized with 30 strokes on ice in a micro-tissue grinder (Spectrum). The homogenate was centrifuged at $1000\times g$ for 5 min at 4°C to eliminate the nuclei and large cellular debris, and the resulting supernatant was centrifuged at $8000\times g$ for 10 min at 4°C. The subsequent supernatant was centrifuged at $100\,000\times g$ for 60 min at 4°C. The pellet was washed in homogenization buffer, and centrifuged again at $100\,000\times g$ for 60 min at 4°C. The resulting pellet was resuspended in PBS buffer (pH 7.4) containing 6 M urea, 0.5% Triton X-100, 0.5% NP-40, 0.1% sodium dodecyl sulfate (SDS), 0.1 mM EDTA and 1 mM EGTA, and was centrifuged at $10\,000\times g$ for 30 min at 4°C. The resulting supernatant was collected as a crude membrane extract.

2.6. Affinity column purification of syntaxin 1C

Syntaxin 1C was purified from cells in an affinity column conjugated with anti-syntaxin 1A monoclonal antibody (14D8). The affinity column was made by coupling 10 mg of the purified anti-syntaxin 1A IgG (14D8) to HiTrap-NHS-activated Sepharose 4FF (Pharmacia). Cytosol was prepared by freezing and thawing 4×10^7 cells in HEPES buffer (10 mM Na^+–HEPES, 135 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 3 μg/ml leupeptin, pH 7.4) and then centrifuging the preparation at $100\,000\times g$; this was then loaded onto the column and washed with 15 column volumes of HEPES. Syntaxin 1C was eluted from the column with 0.1 M glycine–HCl (pH 3.0), and then each fraction was neutralized using 1 M Tris (pH 9.5). The peak protein fraction was immunoblotted as the eluant.

2.7. Immunoblotting and quantitative analysis

Cells on 35-mm dishes (Falcon) were treated with 10% trichloric acid (TCA) on ice for 30 min. The cells were scraped out and centrifuged at 15 000 rpm and 4°C, for 30 min. The precipitate was solubilized with 70 μl of 8 M urea/1% SDS/10 mM Tris–Cl (pH 7.5). After sonication on ice, the samples were centrifuged at 15 000 rpm and 4°C, for 30 min, and the supernatant was obtained. The protein concentration was measured with a DC-protein assay (Bio-Rad).

The samples were separated by 12% SDS–polyacrylamide gel electrophoresis (PAGE), and the blotted PVDF membranes were treated with blocking buffer (5% (w/v) skim milk, 1% (w/v) bovine serum albumin (BSA), PBS) and exposed to anti-syntaxin 1A monoclonal IgG (14D8) (0.1 μg/ml) [21], anti-syntaxin 1C IgG (1 μg/ml), anti-syntaxin 2 polyclonal antibody (0.2 μg/ml) (Stressgen Biotech.), anti-syntaxin 3 polyclonal antibody (1 μg/ml) (Sigma) or anti-syntaxin 4 monoclonal IgG (0.1 μg/ml) (Transduction Lab.). The membranes were exposed to horseradish peroxidase-conjugated anti-mouse IgG coupled to horseradish peroxidase (diluted 1:10 000) (ICN) or anti-rabbit IgG coupled to horseradish peroxidase (diluted 1:5000) (Cappel). Then, the membranes were enhanced with chemiluminescence reagent (Amersham-Pharmacia).

For the peptide suppression experiment, anti-syntaxin 1C antibody was preincubated with 100 μl of 2 mg/ml *pep-1C* peptide at 37°C for 3 h and the preincubated antibody was used in immunoblotting.

For quantitative analysis of syntaxin 1C, after drug stimulation, 15-μg protein samples were loaded and the syntaxin 1C signal was obtained by immunoblotting with anti-syntaxin 1C IgG and 14D8. Then, the membranes used for syntaxin 1C Western blotting were stripped with 0.1 M glycine–HCl (pH 3.0), neutralized, and then treated with anti-α-tubulin IgG (DM1A) (diluted 1:5000) (Sigma) as an internal control. Expression of syntaxins 2, 3 and 4 was also quantitated by the same procedures except using anti-syntaxin 2, 3 and 4 antibody, respectively. The intensities of the immunoblotted signals were measured using NIH Image and the intensity of the syntaxin 1C signal was normalized with that of tubulin.

2.8. Indirect immunofluorescence studies

The cells were cultured on Lab-Tek II 8-well chamber slides (Nunc). Immunostaining was carried out as described previously with slight modification [22]. Briefly, after removing the medium, the dishes were washed twice in PBS. The cells were fixed and permeabilized for 15 min with 4% paraformaldehyde (PFA) containing 0.1% Triton X-100 and PBS. After treatment with blocking solution (5% goat serum, PBS), the cells were incubated with anti-syntaxin 1A IgG (14D8) (1 μg/ml) or anti-syntaxin 1C IgG (10 μg/ml) in PBS containing 5% goat serum and 1% BSA. After washing with PBS, cells were exposed to anti-mouse IgG or anti-rabbit IgG coupled to Alexa 488 (diluted 1:1000) (Molecular Probes). Stained cells were examined using a confocal laser scanning electron microscope (Zeiss LSM 410).

To measure the proportion of cells strongly expressing syntaxin 1C, confocal images were processed using NIH Image. A strongly expressing cell was defined as one exceeding the threshold staining strength of control cells. At least 300 cells were examined in triplicate.

2.9. Statistical analysis

The results are presented as the means ± S.E.M. of triplicate samples from at least two independent experiments. The data were analyzed using one-way analysis of variance. *P* values less than or equal to 0.05 were considered significant.

3. Results and discussion

3.1. PCR analysis of alternatively spliced variants of syntaxin 1

Syntaxin 1A is localized to the plasma membrane of axons and presynaptic terminals in neuronal cells and is involved in the exocytosis of neurotransmitters in neuronal cells [23]. Although syntaxin 1C mRNA is expressed in human fat, skel-

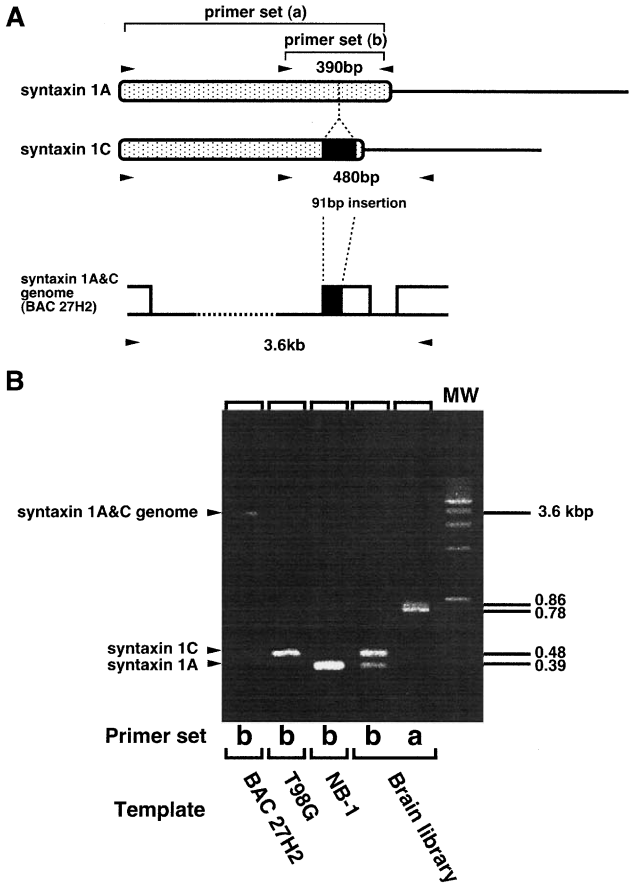

Fig. 1. PCR analysis of alternatively spliced variants of syntaxin 1. A: Schematic representation of the syntaxin 1A variants generated by alternative splicing: coding region (open boxes), syntaxin 1C unique sequence (dark boxes), and 3′-untranslated region (bar). The arrowheads indicate the positions of primer sets a and b. With primer set b, the syntaxin 1A and 1C genes can be discriminated by their sizes (see B). B: PCR identification of alternatively spliced variants of syntaxin 1 in several cell lines. PCR was performed with the two primer sets described in A. A genome containing syntaxin 1A (BAC 27H2) as a negative control, first strand cDNA from T98G astroglioma, first strand cDNA from NB-1 neuroblastoma, and a first strand cDNA library from human brain tissue were analyzed on 1% agarose gels.

etal muscle, liver, and brain [12], its intracellular expression as a protein has not yet been localized. It is well known that neurons express syntaxin 1A. However, it is not known which cell types express syntaxin 1C in the brain.

First, we carried out an RT-PCR analysis using several cell lines derived from human brain. Human neuroblastoma (NB-1 and IMR-32) and astroglioma (T98G, U87MG, U138, U343MGa and SF126) cell lines were used. Human syntaxins 1A and 1C share all but 91 nucleotides of a syntaxin 1C-specific region generated by alternative splicing (see Fig. 1A). Therefore, the PCR analysis was carried out using a pair of PCR primers that distinguished syntaxin 1A and 1C mRNA by molecular weight and could detect genomic contamination during mRNA preparation (Fig. 1A). A genomic DNA clone (BAC 27H2) containing the human syntaxin 1A and 1C genes [24] was used as a control.

We found that all of the astroglioma cell lines studied expressed syntaxin 1C, but not syntaxin 1A, whereas the two human neuroblastoma cell lines expressed only syntaxin 1A. Fig. 1B shows representative results of RT-PCR from human neuroblastoma NB-1 and astroglioma T98G. The 480-bp product expected for syntaxin 1C mRNA was detected only in astroglioma T98G, and not in neuroblastoma NB-1 (Fig. 1B). By contrast, the 390-bp product expected for syntaxin 1A

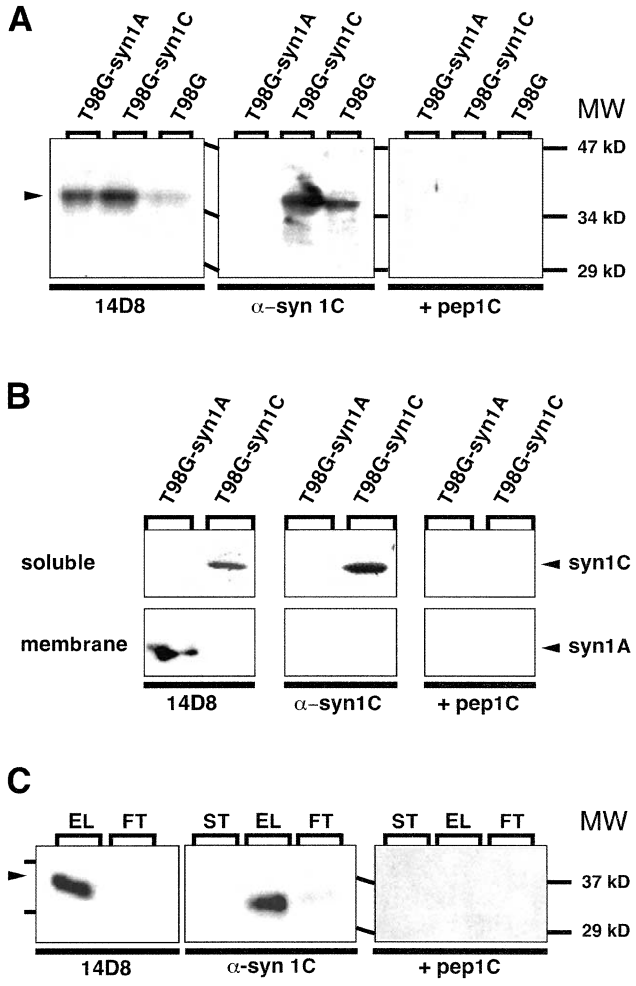

Fig. 2. Identification of syntaxin 1C protein in cells by immunoblotting. A: Syntaxin 1C was expressed in human astroglioma cell line T98G. Syntaxin 1C from T98G parent cells was compared with that from T98G cells expressing exogenous syntaxin 1C. Total homogenates of cells treated with 10% TCA were immunoblotted. Immunoblotting was carried out using anti-HPC-1/syntaxin 1A antibody (14D8), anti-syntaxin 1C antibody (α-syn 1C), or anti-syntaxin 1C antibody in the presence of peptide of the syntaxin 1C-specific region (+pep). The arrowhead indicates HPC-1/syntaxin 1A (M_r 35 000 Da band). A syntaxin 1C signal was detected in the total lysate of T98G cells. B: Immunoblotting of the subcellular fractions in T98G transformants. The soluble and membrane fractions of T98G cells expressing syntaxin 1A or syntaxin 1C were immunoblotted using (left panel) anti-HPC-1/syntaxin 1A antibody (14D8), (middle panel) anti-syntaxin 1C antibody (α-syn 1C), or (right panel) anti-syntaxin 1C antibody in the presence of the peptide of the syntaxin 1C-specific region (+pep 1C). Syntaxin 1C was present in the soluble fraction. C: Immunoblotting of affinity-purified syntaxin 1C in T98G parent cells. The soluble fraction of T98G cells (4×10^7 cells) was affinity-purified using an anti-syntaxin 1A (14D8) antibody-conjugated column and the flow through (FT), eluant (EL), and starting mixture (ST) were immunoblotted. The arrowhead indicates syntaxin 1C. The syntaxin 1C signal was revealed by condensation with affinity purification. This syntaxin 1C signal was blocked by the addition of pep 1C.

was detected only in neuroblastoma NB-1 (Fig. 1B). In the human brain library, both the syntaxin 1A and 1C gene products were detected (Fig. 1B). Therefore, our next experiments sought to detect syntaxin 1C protein in astroglioma cells.

3.2. Identification of syntaxin 1C protein in T98G cells by immunoblotting

In order to determine the specificity of syntaxin 1C antibody, we carried out an immunoblot analysis of recombinant syntaxins 1A and 1C expressed by *Escherichia coli*. An antibody for the N-terminal region of syntaxin 1A (14D8) recognized both syntaxins 1A and 1C, whereas an antibody raised against the C-terminal unique region of syntaxin 1C specifically recognized syntaxin 1C (data not shown).

We next investigated the expression of syntaxin 1C protein in astroglioma cells. T98G transformants expressing syntaxin 1A or 1C were used as controls. Fig. 2A shows that syntaxin 1C protein is present in T98G astroglioma cells. The signal of the anti-syntaxin 1C antibody was suppressed by preincubation with syntaxin 1C-specific peptide (*pep-1C*) (Fig. 2A). Interestingly, the apparent molecular weight of syntaxin 1C on SDS–PAGE was almost the same as that of syntaxin 1A, although the theoretical molecular weights of syntaxins 1A and 1C differ (Fig. 2A). This result confirmed the report of Jagadish et al. [12]. However, the amount of syntaxin 1C protein expressed in T98G cells appeared much lower than that of syntaxin 1A protein in PC12h, which strongly expresses syntaxin 1A protein (data not shown).

3.3. Intracellular localization of syntaxin 1C in T98G transformants

In order to localize syntaxin 1C within astroglioma cells, we subfractionated T98G or U87MG transformants of syntaxin 1C to obtain soluble and crude membrane fractions. Exogenous syntaxin 1C protein was detected in the soluble fraction by Western blotting, whereas syntaxin 1A protein was detected only in the crude membrane fraction (Fig. 2B).

We tried to detect syntaxin 1C in T98G parent cells after condensation using affinity column chromatography, since the amount of native syntaxin 1C expressed is too small to analyze. The soluble fraction of T98G (4×10^7 cells) was purified in an affinity column coupled with anti-syntaxin 1A monoclonal antibody (14D8), and immunoblotted. As shown in Fig. 2C, a syntaxin 1C signal was detected in parent cells using both 14D8 and syntaxin 1C antibody, but not when anti-syntaxin 1C antibody was preincubated with syntaxin 1C-specific peptide (*pep-1C*) (Fig. 2C). A similar result was observed in U87MG cells (data not shown). These results show that syntaxin 1C protein was present in the soluble fraction, but not in the membrane fraction, unlike syntaxin 1A protein.

Syntaxin 1C possesses the same N-terminal domain (1–225 amino acid residues) as syntaxin 1A, however, loses TMD and the latter half of H3 domain which were converted into the novel C-terminal proline-rich region of 35 residues generated by alternative splicing. Since the H3 domain (191–267 residues in syntaxin 1A) is necessary for interaction with most of SNAREs [25–28], the C-terminal of syntaxin 1C would lose capacity for binding with SNAREs except n-Sec/Munc18 and SNAP-25. In addition, we found that syntaxin 1C is detected only in soluble fraction (Fig. 2B, C), suggesting that syntaxin 1C did not interact with, at least, other membrane-bound SNAREs in astroglioma cells.

3.4. Phorbol-ester stimulation induced expression of syntaxin 1C protein

In order to determine whether the expression of syntaxin 1C is regulated via any signal transduction system, T98G cells were stimulated with forskolin and PMA, which activate protein kinase A (PKA) and PKC, respectively.

As shown in Fig. 3A, RT-PCR analysis confirmed that stimulation of T98G cells with 10 μM PMA appeared to increase the expression of syntaxin 1C mRNA (480 bp signal). However, syntaxin 1A signal (390 bp) was not observed in the

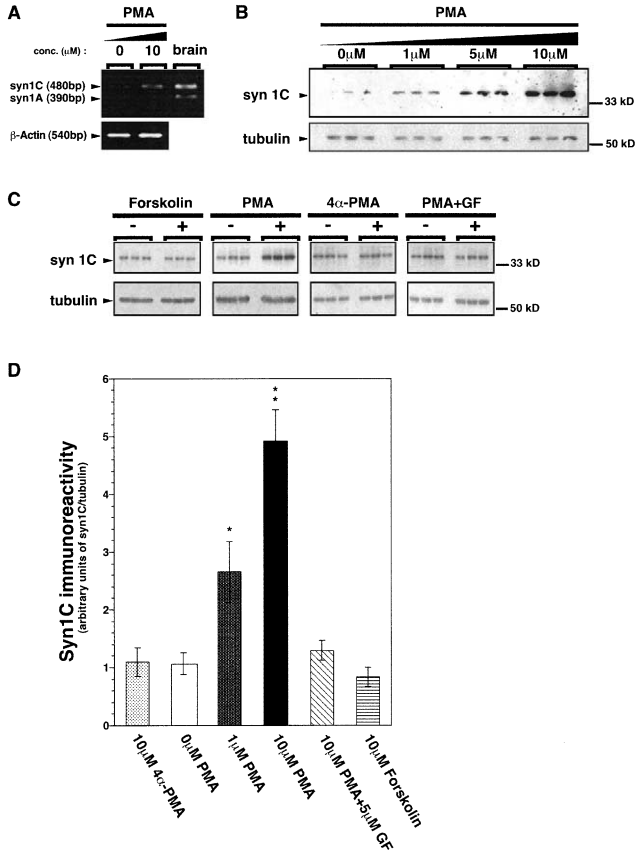

Fig. 3. Expression of syntaxin 1C in T98G astroglioma cells treated with PMA and forskolin. A: RT-PCR analysis of syntaxin 1C expression in T98G cells treated for 12 h with 10 μM PMA. RT-PCR was performed as shown in Fig. 1. The syntaxin 1C PCR product (480 bp), but not that of syntaxin 1A (390 bp), appeared to increase (3.09 ± 0.55-fold) in T98G cells with PMA stimulation. However, the syntaxin 1A was not detected in control nor PMA-stimulated cells. A first strand cDNA library from human brain tissue (brain) was also analyzed to distinguish syntaxins 1A and 1C. B: PMA dose dependence of syntaxin 1C expression. T98G cells were treated for 12 h with 0–10 μM PMA. 15 μg of TCA lysates were immunoblotted with 14D8 (35 kDa). The membranes were then immunoblotted with anti-α-tubulin IgG (55 kDa). C: Expression of syntaxin 1C in T98G cells treated for 12 h with 10 μM forskolin, 4α-PMA, PMA, or PMA plus 5 μM PKC inhibitor, GF. D: Quantification of the syntaxin 1C signal in cells treated with drugs. Quantitative densitometric analysis was performed using NIH Image. The values are expressed in arbitrary units of syntaxin 1C density relative to tubulin density. The results are the means ± S.E.M. of nine independent experiments. The syntaxin 1C expression increased in T98G cells with PMA stimulation. The results were compared to those for 0 μM PMA (0.1% dimethylsulfoxide (DMSO)) statistically. 0 μM PMA = 1.07 ± 0.18, 4α-PMA = 1.09 ± 0.25 ($P > 0.05$), 1 μM PMA = 2.65 ± 0.52 (*$P < 0.01$), 10 μM PMA = 4.91 ± 0.56 (**$P < 0.001$), forskolin = 0.84 ± 0.16 ($P > 0.05$), PMA+GF = 1.29 ± 0.17 ($P > 0.05$).

control nor PMA-stimulated cells by PCR (Fig. 3A). Furthermore, quantitative immunoblot analysis demonstrated that syntaxin 1C protein expression was enhanced about two-fold with 1 μM PMA and about five-fold with 10 μM PMA stimulation (Fig. 3B, C, D), whereas the amount of plasma membrane syntaxins (syntaxins 2, 3 and 4) was not changed by PMA stimulation (data not shown). This effect of PMA at high concentration suggests that a low-responsive isoform of conventional or novel PKC activated by PMA participates in the induction of syntaxin 1C in T98G cells [29]. By contrast, in the presence of 10 μM forskolin, syntaxin 1C expression did not change significantly (0.84 ± 0.16) (Fig. 3C, D). 10 μM 4α-PMA, a non-effective analog of PMA, had no effect (1.09 ± 0.25) (Fig. 3C, D).

We also carried out indirect immunofluorescence analysis to study the effect of PMA. In control cells, intracellular expression of syntaxin 1C was generally weak, although a few cells showed high expression (Fig. 4A). We found that PMA enhanced the expression of syntaxin 1C in most cells and the number of cells strongly expressing it increased with PMA stimulation (Fig. 4A, B). This phenomenon was not observed in PC12h cells expressing syntaxin 1A and not syntaxin 1C (data not shown). By contrast, forskolin and 4α-PMA were not effective (Fig. 4A, B). At present, the reason for the different responses by T98G cells is not clear.

Furthermore, immunoblot analysis showed that upregulation of syntaxin 1C expression increased for at least 24 h during PMA stimulation (data not shown) and indirect im-

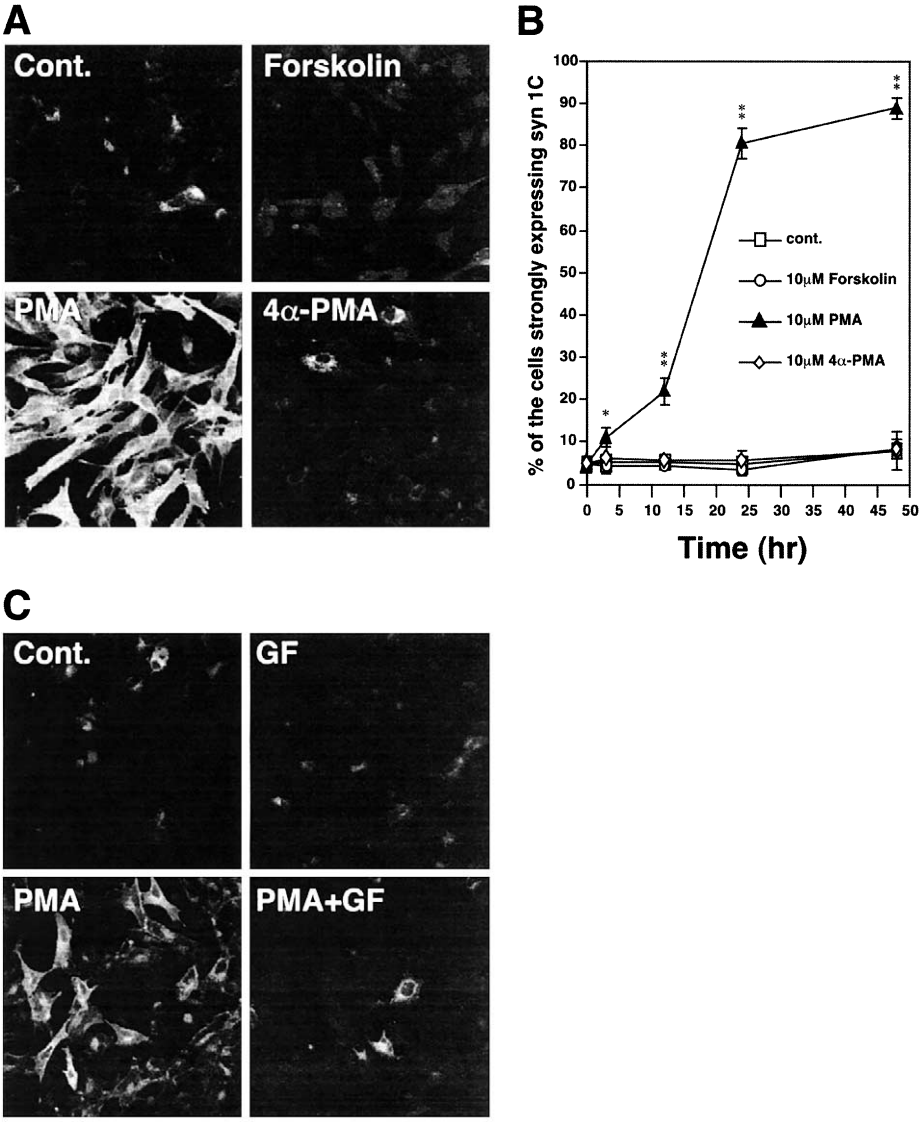

Fig. 4. PMA stimulation of the intracellular expression of syntaxin 1C is dependent on stimulation time. A: Intracellular expression of syntaxin 1C in T98G cells treated with PMA and forskolin. Indirect immunofluorescence analysis of T98G astroglioma cells treated with 10 μM forskolin, 10 μM PMA or 10 μM 4α-PMA for 24 h. B: Proportion of cells strongly expressing syntaxin 1C with drug treatment. The results are given as percentages (means ± S.E.M.), $n = 3$. The number of cells strongly expressing syntaxin 1C with PMA treatment exceeded that for control cells or with forskolin or 4α-PMA at 3 h (*$P < 0.05$), 12, 24, or 48 h (**$P < 0.01$). C: PKC inhibitor suppresses enhancement of syntaxin 1C expression by PMA. T98G astroglioma cells were treated with 1 μM PMA for 12 h (PMA). In order to know the effect of PKC inhibitor, the cells were pretreated with 5 μM GF for 3 h and further incubated with 5 μM GF alone (GF) or with both 1 μM PMA and 5 μM GF (PMA+GF) for 12 h.

munofluorescence analysis showed that the number of cells strongly expressing syntaxin 1C increased during PMA stimulation (Fig. 4B). This slow, sustained activation of syntaxin 1C by PMA suggests an indirect process, requiring de novo, ongoing protein synthesis or suppressing protein degradation.

To ensure that the increase in syntaxin 1C expression with PMA was a specific effect, we treated the cells with 5 μM GF, a bisindolylmaleimide, which inhibits the activity of all three PKC isoforms (conventional, novel, atypical) by competing for the ATP binding site [30,31]. Figs. 3C, 3D, and 4C show that syntaxin 1C expression was no longer enhanced by treatment with this PKC inhibitor. These results imply that syntaxin 1C expression is regulated via the PKC signaling pathway. Since it has been reported that the expression of syntaxin 1A mRNA is upregulated by forskolin, but not by PMA [15], perhaps syntaxin 1A and 1C expressions are regulated in different manners in neuronal and glial cells, respectively. From the viewpoint of the regulation of gene transcription, respective assay systems using neuroblastoma and astroglioma cells expressing syntaxins 1A and 1C will be useful for studying the mechanisms regulating syntaxin 1 expression. Since it was known that the syntaxin family participates in the intracellular membrane transport mechanism, it is possible that syntaxin 1C participates in intracellular transport. However, at present, the physiological function and expression mechanism of syntaxin 1C are unknown, and further studies are clearly necessary.

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