Protein Kinase C (PKC)-promoted Endocytosis of Glutamate Transporter GLT-1 Requires Ubiquitin Ligase Nedd4-2-dependent Ubiquitination but Not Phosphorylation*

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Glutamate transporter-1 (GLT-1) is the main glutamate transporter in the central nervous system, and its concentration severely decreases in neurodegenerative diseases. The number of transporters in the plasma membrane reflects the balance between their insertion and removal, and it has been reported that the regulated endocytosis of GLT-1 depends on its ubiquitination triggered by protein kinase C (PKC) activation. Here, we identified serine 520 of GLT-1 as the primary target for PKC-dependent phosphorylation, although elimination of this serine did not impair either GLT-1 ubiquitination or endocytosis in response to phorbol esters. In fact, we present evidence indicating that the ubiquitin ligase Nedd4-2 mediates the PKC-dependent ubiquitination and down-regulation of GLT-1. Overexpression of Nedd4-2 increased the ubiquitination of the transporter and promoted its degradation. Moreover, phorbol myristate acetate enhanced Nedd4-2 phosphorylation and the formation of GLT-1:Nedd4-2 complexes, whereas siRNA knockdown of Nedd4-2 prevented ubiquitination, endocytosis, and the concomitant decrease in GLT-1 activity triggered by PKC activation. These results indicate that GLT-1 endocytosis is independent of its phosphorylation and that Nedd4-2 mediates PKC-dependent down-regulation of the transporter.

The glutamate released by neurons into the synaptic cleft is inactivated by glutamate transporters, thereby preventing the excessive stimulation of glutamate receptors and any resulting neurotoxicity. This inactivation is accomplished by five glutamate transporters from the SLC1 gene family, although one of them is responsible for up to 90% of extracellular glutamate clearance in the forebrain, GLT-1¹ (EAAT2 in humans) (1). There are multiple splice variants of GLT-1 that differ in their N- and C-terminal sequences, and despite being mainly expressed in astrocytes, it is one of the most abundant proteins in the brain (2, 3). The importance of GLT-1 in maintaining glutamate levels below the neurotoxic threshold has been demonstrated in animals with diminished levels of this transporter, which are highly vulnerable to excitotoxic insults and seizures (1). GLT-1 is a highly regulated transporter modulated by changes in both transporter expression and protein activity. The activity of GLT-1 is affected by several effectors, including free radicals, arachidonic acid, protein kinase C (PKC), protein kinase A, and serum- and glucocorticoid-inducible kinases (SGK1, SGK2, and SGK3) (4–8).

Like many other membrane transporters, GLT-1 protein trafficking to and from the plasma membrane is a means to rapidly regulate its activity (3, 9). PKC activation by phorbol esters promotes rapid changes in GLT-1 transporter activity. Thus, in primary cultures of astrocytes, mixed cultures of neurons and glia, and cell lines like glioblastoma C6 and MDCK, phorbol esters down-regulate GLT-1, which is endocytosed from the plasma membrane into intracellular compartments (6, 7, 10, 11). Indeed, in C6 cells, this process depends on a 43-residue fragment in the C-terminal tail of the transporter (6). It is thought that GLT-1 endocytosis in response to phorbol esters depends on the clathrin pathway (10, 12) with the endocytosed

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² The abbreviations used are: GLT-1, glutamate transporter-1; ENaC, epithelial sodium channel; PMA, phorbol myristate acetate; SGK, serum- and glucocorticoid-inducible kinase; MDCK, Madin-Darby canine kidney; DAT, dopamine transporter; Nedd4-2, neural precursor cell-expressed, developmentally down-regulated 4-2; NHS, N-hydroxysulfosuccinimide; HECT, Homologous to E6AP COOH terminus.
transporter then targeted for lysosomal degradation (12). Nevertheless, recent observations support a prominent role for the flotillin-1 endocytosis pathway, although it is unclear whether this is a cell-specific phenomenon or whether there is some kind of interaction between the clathrin and flotillin-1 pathways (13). PKCα has been shown to associate physically with GLT-1 and to phosphorylate the transporter. Significantly, GLT-1 down-regulation is partially disrupted by the mutation of serine 486 (6, 14), although a cause-and-effect relationship between the phosphorylation of GLT-1 and its endocytosis has yet to be formally proven because phosphorylation in response to phorbol esters is not disrupted in the S486A mutant. Another transporter that is also down-regulated by PKC is the dopamine transporter (DAT), which belongs to the family of sodium- and chloride-dependent neurotransmitter transporters. This transporter is internalized in response to phorbol esters even after deletion of the putative phosphorylation sites (15), although this process appears to require the phosphorylation of the adaptor flotillin-1 rather than that of DAT (13).

Recent evidence has shown that PKC-dependent endocytosis of GLT-1 depends on the ubiquitination of the transporter at the cell surface (11, 16), suggesting that ubiquitin might act as a platform to recruit the endocytic machinery to GLT-1. Ubiquitination of the transporter occurs at the lysines located in the C-terminal tail of GLT-1, although the ubiquitin ligase responsible for that modification remains unidentified. Ubiquitination involves the addition of the polypeptide ubiquitin to some free amino groups in proteins via an isopeptide bond, mainly to the ε-amino of lysines, which is catalyzed by the sequential action of three enzymes, E1, E2, and E3. E3 ligases transfer ubiquitin to the specific substrate, and they are classified into two main families, RING (about 600 ligases) and HECT (about 30 ligases) (17). One member of the HECT family, neural precursor cell-expressed, developmentally down-regulated 4-2 (Nedd4-2), has been implicated in the ubiquitination of many mammalian transporters and channels (18–26), including neurotransmitter transporters like the dopamine transporter (27) and the glutamate transporter EAAT2 (8). In addition to the catalytic HECT domain, Nedd4-2 contains four WW domains that typically interact with PPXY motifs present in its substrates (28–33). However, many Nedd4-2 substrates do not have the PPXY motif and interact indirectly through intermediate proteins (17, 25). For instance, the yeast homologue of Nedd4-2, Rsp5, binds to several transporters through β-arrestin-like adaptor proteins called α-arrestins (24, 34).

In this report, we identify the ubiquitin ligase Nedd4-2 as a mediator of the GLT-1 endocytosis that is triggered by PKC activation. PKC promotes the phosphorylation of Nedd4-2, its association with GLT-1, and the subsequent ubiquitination of the transporter that precedes its endocytosis. Although GLT-1 is also phosphorylated at serine 520 after PKC activation, this phosphorylation is not necessary for GLT-1 endocytosis.

EXPERIMENTAL PROCEDURES

Materials—[3H]glutamate, protein standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE Rainbow markers), and the Enhanced Chemiluminescence (ECL) Western blotting detection reagents were all obtained from Amersham Biosciences. Lipofectamine™ 2000 and pcDNA3 plasmid were purchased from Invitrogen; TrueFect-Lipo™ was from United Biosystems (Rockville, MD); and phenylmethanesulfonyl fluoride (PMSF), the Expand High Fidelity PCR system (Tag polymerase), and all restriction enzymes were obtained from Roche Applied Science. The QuikChange Site-Directed Mutagenesis kit was from Stratagene (La Jolla, CA), nitrocellulose sheets were from Bio-Rad, and fetal calf serum was supplied by Invitrogen. The monoclonal mouse anti-hemagglutinin (HA) (clone 12CA5) was prepared at the microscopy service of the Centro de Biología Molecular (Madrid, Spain), the Alexa Fluor 488- or Alexa Fluor 555-coupled goat anti-rabbit and goat anti-mouse secondary antibodies were obtained from Molecular Probes (Eugene, OR), and the mouse monoclonal anti-ubiquitin (P4D1) was from Santa Cruz Biotechnology (Santa Cruz, CA). The agarose-conjugated anti-mutilubiquitin was purchased from MBL International (Woburn, MA). Vectashield was obtained from Vector Laboratories (Burlingame, CA), and EZ-Link Sulfo-NHS-SS-Biotin was from Pierce. The pGEM-T easy cloning vector was purchased from Promega (Madison, WI), and the oligonucleotides used were synthesized by Isogen Life Science (Utrecht, Netherlands). All other chemicals were obtained from Sigma.

Cell Growth and Transfection—COS-7 and MDCK cells (American Type Culture Collection) were grown in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in an atmosphere of 5% CO₂. Transient expression in COS-7 or MDCK cells was achieved using TrueFect-Lipo or Lipofectamine 2000, respectively, according to the manufacturers’ instructions. The cells were incubated for 48 h at 37 °C and then analyzed biochemically or by immunofluorescence and/or in transport assays.

Plasmid Constructs—The various GLT-1 mutants were prepared using rat GLT-1 in pcDNA3 as a template (derived from the GLT-1 clone obtained from Dr. B. I. Kanner) and the QuikChange Site-Directed Mutagenesis kit according to the manufacturer’s instructions. Previously, an HA tag was included at the N terminus of the transporter by PCR. The inclusion of this tag had no effect on the activity or on the subcellular distribution of the protein. Nedd4-2-yellow fluorescent protein (YFP) was obtained in two steps, first using the Ypet variant of YFP (kindly donated by Dr Daugherty; Ref. 35) as a template for PCR amplification with specific oligonucleotides containing restriction sites for BamHI and EcoRI (forward and reverse, respectively). The amplified fragment was ligated into the BamHI/EcoRI sites of pcDNA3, and the full-length Nedd4-2 was then amplified by PCR using the cDNA clone MGC:78116 as the template and specific oligonucleotides containing restriction sites for HindIII and BglIII (forward and reverse, respectively). The stop codon was omitted in the reverse primer, and the product was ligated in-frame into the HindIII/BamHI sites of YFP-pcDNA3. For electrophysiological recordings, the cDNAs for GLT-1, Nedd4-2, AIP4, and AIP2 were subcloned into the vector pSP64T, which contains the 5′- and 3′-UTRs of the Xenopus laevis β-globin gene (provided by Dr. Carmen Montiel, Universidad Autónoma de Madrid). All constructs and mutants were confirmed by sequencing.
Expression in Xenopus Oocytes—The cDNAs cloned into pSP64T were linearized with XbaI, and the cRNAs were transcribed with SP6 polymerase and capped with 5′-7-methylguanosine using the mMESSAGE mACHINE SP6 RNA kit (Ambion Inc.). X. laevis frogs were obtained from Xenopus Express (France), and oocytes were harvested from X. laevis anesthetized in 0.1% (w/v) Tricaine methanesulfonate solution in tap water. All these procedures were performed in accordance with the Spanish and European guidelines for the prevention of cruelty to animals. The follicular membrane was removed by incubation in a medium (90 mM NaCl, 1 mM KCl, 1 mM MgCl₂, 0.3 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 2.4 mM NaHCO₃, 10 mM HEPES (pH 7.4)), and transport or electrophysiological experiments were carried out 5 days later.

Two-microelectrode Voltage Clamp Recordings of Xenopus Oocytes—Electrophysiological recordings were obtained after incubating the injected oocytes at 18 °C in standard oocyte solution (100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH adjusted to 7.5 with HCl). A two-electrode voltage clamp was used to measure and control the membrane potential and to monitor the capacitative currents using Axoclamp 900A (Axon Instruments); the two electrode signals were digitized by a Digidata 1440A (Axon Instruments), and both instruments were controlled by the pCLAMP software (Axon Instruments). The results were analyzed by Clampfit 10.2 software (Axon Instruments). The recordings were performed at room temperature (RT) using standard micropipettes filled with 3 M KCl (resistance varied between 0.5 and 2 megaohms). Oocytes were held at −40 mV, and currents were subjected to low pass filtering at 100 Hz.

Metabolic Labeling and Immunoprecipitation—COS-7 cells were plated on p35 dishes and transfected with Lipofectamine-PLUS according to the manufacturer’s instructions. Two days later, the cells were incubated in phosphate-free DMEM for 2 h and then metabolically labeled with 0.06 mCi/ml ³²P-labeled inorganic phosphate (H₂¹³⁰P). After labeling for 3.5 h, the cells were treated with phorbol ester (0.5 μg/ml) or vehicle for 20 min, rinsed with Tris-buffered saline solution, and harvested in immunoprecipitation lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA, 50 mM NaF, 0.2 mM NaVO₄). After incubation for 30 min at 4 °C, cell debris was removed by centrifugation at 12,000 × g for 10 min, and the supernatant was used for immunoprecipitation. The immunoprecipitated material was resolved by electrophoresis as described below, and after drying, the gels were submitted to autoradiography.

Immunoprecipitation—Transiently transfected cells were solubilized in radioimmuno precipitation assay buffer (50 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 2 mM EDTA, 0.1 mM DTT). The solubilized material was centrifuged at 10,000 × g for 20 min, and the supernatant was incubated overnight at 4 °C with the desired antibodies (2 μg/ml). Subsequently, 40 μl of protein A cross-linked to agarose beads (Sigma) was added, and the mixture was incubated for 1 h at 4 °C with constant rotation. The beads were washed five times with ice-cold lysis buffer before adding SDS-PAGE sample buffer to each sample (25 μl). The bound proteins were dissociated from the beads by heating at 92 °C for 3 min before they were resolved on 7.5% gels by SDS-PAGE as described below. In some experiments, ubiquitinated proteins were isolated using agarose-conjugated anti-multiubiquitin following the manufacturer’s instructions.

Electrophoresis and Immunoblotting—SDS-PAGE was performed on 7.5% polyacrylamide gels in the presence of 2-mercaptoethanol. After electrophoresis, the protein samples were transferred to nitrocellulose membranes in a semidy electrophoretic blotting system at 1.2 mA/cm² for 2 h (LKB) using a transfer buffer containing 192 mM glycine and 25 mM Tris-HCl (pH 8.3). Nonspecific binding to the membrane was blocked by incubating the filter with 5% nonfat milk protein in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl for 4 h at 25 °C. The membrane was then probed overnight at 4 °C with the diluted primary antibody (anti-HA or anti-ubiquitin), which was detected after washing with an anti-mouse IgG peroxidase-linked secondary antibody. The labeled bands were visualized by ECL and quantified by densitometry on a GS-710 calibrated imaging densitometer from Bio-Rad with Quantity One software by using film exposures in the linear range. For proteins labeled with ³²P, the dried gels were rehydrated in 50 mM ammonium bicarbonate for 16 h, and the proteins were transferred to polyvinylidene fluoride (PVDF) membranes before they were incubated with the primary and secondary antibodies.

Transport Assays—[³H]Glutamate transport in transfected cells was measured as described previously (36).

Protein Determination—Protein concentrations were determined with the Bio-Rad Protein Determination kit using bovine serum albumin (BSA) as the standard.

Cell Surface Biotinylation—Cells were plated at 50% confluence in 60-mm cell culture plates and transfected as indicated above. After 2 days, the cells were washed with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄ (pH 7.3)), and the cell surface proteins were labeled for 20 min at 4 °C by incubating them in a 1-ml solution containing the non-permeable Sulfo-NHS-SS-Biotin reagent (1 mg/ml in PBS). The cells were then washed with 2 ml of PBS plus 100 mM lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM HEPES-Tris, 0.25% sodium deoxycholate, 1% Triton X-100, 0.1% SDS (pH 7.4)), and the lysate was cleared by centrifugation at 14,000 × g for 10 min. The biotinylated proteins were finally recovered by incubating the cleared lysate for 2 h at RT with streptavidin-agarose beads. After washing the beads three times with 1 ml of the lysis buffer, the protein bound to the beads was eluted in 2× Laemmli sample buffer, separated by SDS-PAGE, and analyzed in Western blots. Biotinylated GLT-1 was visualized with anti-HA. To determine ubiquitinated GLT-1 in the biotinylated protein fraction, proteins were eluted from beads with 1% SDS (92 °C for 5 min). Subsequently, SDS was diluted to 0.1%, and
HA-GLT-1 was immunoprecipitated and probed in Western blots with anti-ubiquitin and anti-HA antibodies.

Immunofluorescence in Cultured Cells—MDCK cells grown on glass coverslips were transfected with expression vectors using Lipofectamine 2000 according to the manufacturers’ instructions. Two days later, the cells were rinsed with PBS and fixed for 20 min with 4% paraformaldehyde in PBS. After washing with PBS, the cells were permeabilized and blocked at RT for 1 h in PBS containing 1% BSA and 0.02% digitonin. The cells were then incubated for 1 h at RT with anti-HA in PBS containing 0.1% BSA and 0.02% digitonin. After three washes with PBS, the cells were incubated with Alexa Fluor 488- (1:250) or Alexa Fluor 594-conjugated goat anti-mouse secondary antibodies for 1 h at RT. Finally, the cells were washed exhaustively with PBS, and the coverslips were mounted in Vectashield. Antibody staining was visualized, and the images were captured on a confocal MicroRadiance (Bio-Rad) coupled to an Axioscop2 microscope (Carl Zeiss, Jena, Germany).

RNA-mediated Interference—Small-interfering RNA (siRNA) oligonucleotides were purchased from Ambion, and a Silencer predesigned siRNA duplex for Nedd4-2 (siRNA identification number s23570) was used. The siRNAs (30 pmol of each siRNA) were transfected into COS-7 cells (0.5 × 10⁶ cells) by nucleofection with the Amxaca electroporation system using the W-01 program of the Nucleofector device according to the manufacturer’s instructions (Lonza Group Ltd., Switzerland). The cells were then plated at 80% confluence, and 24 h later, the cells were transfected with HA-GLT-1 using lipofection. Experiments were performed 24 h later. siRNA nucleofection efficiency was over 95% as evaluated with the maxGFP™ positive control (Lonza Group Ltd.).

RESULTS

Effect of PMA on GLT-1 Phosphorylation—The phorbol ester PMA promotes PKC-dependent endocytosis of GLT-1 in several experimental systems, although it is unclear whether phosphorylation of the transporter is associated with this process. Sequence analysis using NetPhos 2.0 software (37) indicated that seven serines within the N- and C-terminal tails of GLT-1 are potential targets for phosphorylation (Ser-24, -25, -496, -498, -520, -531, and -563), although they did not match the consensus sequence for PKC phosphorylation. Consequently, a construct was produced in which these seven serines were mutated to alanines, and its phosphorylation in response to PMA was analyzed after expression in COS-7 cells (Fig. 1, upper panels). PMA increased the incorporation of ³²P into several bands that were immunoprecipitated with anti-HA antiserum from extracts of cells transiently transfected with HA-GLT-1WT (Fig. 1, lanes 3 and 4). In the presence of PMA, the incorporation of ³²P in cells transfected with the mutant HA-GLT-1 7SA was equivalent to that in the untreated control (Fig. 1, lanes 5 and 6). Furthermore, the phosphorylation of a mutant lacking the five C-terminal serines (HA-GLT-1 5SA) after PMA treatment was also similar to that in control untreated cells (Fig. 1, lanes 7 and 8). To determine the contribution of each of the five C-terminal serines to PMA-mediated phosphorylation of
GLT-1, they were each reintroduced in the HA-GLT-1 5SA mutant, and the mutants were assayed as above. Only reintroduction of Ser-520 restored the incorporation of $^{32}$P when the transfected cells were exposed to PMA (Fig. 1, lanes 9 and 10). Indeed, elimination of this serine alone in the wild type protein abolished the increase in phosphorylation of GLT-1 induced by PMA (Fig. 1, lanes 11 and 12), whereas elimination of the other serines had no significant effect on $^{32}$P incorporation (data not shown). In all cases, the radiolabeled bands corresponded to the transfected GLT-1 because they were not observed in mock-transfected cells (Fig. 1, lanes 1 and 2). Moreover, the differences in labeling were not due to differences in protein expression as evident when each gel determining $^{32}$P incorporation was rehydrated, immunoblotted, and probed with anti-HA antibodies (Fig. 1, lower panels). Comparable immunoreactivity was observed in the different extracts independently of the construct transfected or treatment. Together, these assays indicate that serine 520 is the prominent residue in GLT-1 phosphorylated following PKC activation.

**Phosphorylation of GLT-1 Is Not Required for Its Endocytosis**—After identifying the target residue for GLT-1 phosphorylation, we explored the role of this residue in its endocytosis. The different serine-to-alanine mutants were transiently transfected into COS cells, and the response to PMA was followed by immunofluorescence microscopy (Fig. 2). Similarly to HA-GLT-1WT, constructs HA-GLT-1 7SA, HA-GLT-1 S520A and HA-GLT-1 5SA A520S were concentrated at the cell surface of control cells (treated with the DMSO vehicle alone) (Fig. 2, A, C, E, and G, respectively). Subsequently, incubation with PMA for 30 min promoted internalization of the wild type and mutated forms of HA-GLT-1 into intracellular compartments independently of the presence or absence of serine 520 (Fig. 2, B, D, F, and H). As a negative control and consistent with previous reports, a ubiquitination-defective mutant lacking lysines in the C terminus was not endocytosed in response to PMA (Fig. 2, compare I and J). Although COS cells are not an optimal system for immunofluorescence studies due to high intracellular retention of overexpressed proteins, similar results were obtained when these experiments were repeated in transfected primary astrocytes or in the epithelial cell line MDCK (supplemental Figs. S1 and S2). The effect of these mutations was quantified by measuring $[^3]$H]glutamate uptake into transfected COS cells (Fig. 3A). PMA reduced transport by 39 ± 3% in cells transfected with HA-GLT-1WT, and a similar inhibition was observed in cells transfected with HA-GLT-1 7SA, HA-GLT-1 5SA, or HA-GLT-1 5SA A520S as well as in mutant HA-GLT-1 S520A.

Consistent with the idea that ubiquitination but not phosphorylation is required for GLT-1 endocytosis, we found that HA-GLT-1 7SA was still ubiquitinatated in response to PMA at levels similar to that of HA-GLT-1WT (Fig. 3, B, left panel, and quantification in C). Reprobing of these blots with anti-HA antiserum confirmed that these constructs had similar levels of expression (Fig. 3B, right panel).

**Ubiquitin Ligase Nedd4-2 Promotes Ubiquitination and Degradation of GLT-1**—The ubiquitin ligase Nedd4-2 is thought to down-regulate the activity of the human EAAT2 in *Xenopus* oocytes, although the molecular mechanisms underlying this effect remain unclear (8). Similarly to the human transporter, glutamate promoted sodium currents in *Xenopus* oocytes injected with rat GLT-1 mRNA, and these were reduced by 76 ± 6% when oocytes were co-injected with Nedd4-2 mRNA, whereas the ubiquitination-defective mutant of Nedd4-2 (C822S) only reduced such currents by 8 ± 3% (Fig. 4A). By contrast, the AIP2 and AIP4 ubiquitin ligases were ineffective in modulating GLT-1-mediated currents (Fig. 4A). In mammalian cells (COS-7), co-expression of GLT-1 and Nedd4-2 reduced the uptake of radioactive glutamate to 43 ± 5% of the control levels (Fig. 4B), and as observed in *Xenopus* oocytes, neither
AIP2 nor AIP4 had a significant effect on glutamate uptake (Fig. 4B). To test the effect of Nedd4-2 on the ubiquitination of GLT-1, a YFP-tagged form of Nedd4-2 (Nedd4-2-YFP) and HA-GLT-1 were co-expressed in COS cells, and the transporter immunoprecipitated with anti-HA antiserum was analyzed in a Western blot. When the blots were probed with an antiserum against ubiquitin, an increase in the amount of ubiquitin incorporated into GLT-1 was evident in the presence of Nedd4-2 (Fig. 5, A, WB: αUb, and quantification in C). In accordance with the data obtained in the transport assay in Xenopus oocytes, there was a decrease in the amount of GLT-1 when the blot was reprobed with anti-HA antibodies, a decrease that was also observed in the corresponding lysate lane (Fig. 5, A and quantification in C). Previous studies have shown the importance of a cluster of C-terminal lysines in regulating the PMA-induced endocytosis of GLT-1. When the ubiquitination of GLT-1 was analyzed, there was a notable decrease in the amount of ubiquitin incorporated by Nedd4-2 into a mutant in which the seven C-terminal lysines had been mutated to arginines (mutant 7KR) when compared with HA-GLT-1WT (Fig. 5, B and quantification in C). Nevertheless, the ubiquitination observed in this mutant suggests that Nedd4-2 can target additional lysines. In agreement with these data, the 7KR mutant was less sensitive to the presence of Nedd4-2 in glutamate uptake assays (uptake was reduced by 22% ± 3%; Fig. 5D). Together, these results indicate that Nedd4-2 can ubiquitinate GLT-1, thereby decreasing the amount of GLT-1 in the cell.

PMA Promotes Association of Nedd4-2 with GLT-1—Nedd4-2 is known to interact either directly or indirectly with target proteins, and hence, we assessed whether it associated...
with GLT-1 in cells co-expressing HA-GLT-1 and Nedd4-2-YFP and maintained in the presence or absence of PMA. GLT-1 was immunoprecipitated from extracts of these cells, and the material recovered was immunoblotted and probed with anti-YFP antibodies. An immunoreactive band of 150 kDa was observed only when both proteins were co-expressed in PMA-treated cells but not in the control cells (mock-transfected or transfected with GLT-1 or Nedd4-2-YFP alone) (Fig. 6A, asterisk). In the reverse immunoprecipitation experiment with anti-YFP antiserum, a small amount of HA-GLT-1 was immunoprecipitated in control cells that increased following PMA treatment (Fig. 6B, asterisk).

**PMA Promotes Phosphorylation of Nedd4-2**—Nedd4-2 is phosphorylated by several kinases, and phosphorylation regulates its association/dissociation with target proteins. Thus, we explored whether it was phosphorylated when PKC was activated by phorbol esters. COS cells were transiently transfected with Nedd4-2-YFP, metabolically labeled with 32P, and treated with PMA or the vehicle alone for 30 min. When Nedd4-2-YFP was immunoprecipitated and analyzed by gel electrophoresis, the basal phosphorylation of a 150-kDa band corresponding to Nedd4-2-YFP increased upon PMA treatment (Fig. 7).

**Nedd4-2 Knockdown Blocks Action of PMA on GLT-1**—To further evaluate whether Nedd4-2 is involved in the inhibitory effect of PMA on GLT-1, we assessed the sensitivity of GLT-1 to PMA when this ubiquitin ligase was abrogated. Synthetic siRNA was introduced into COS cells by nucleofection to knock down Nedd4-2 expression. Subsequently, cells were also transfected with HA-GLT-1 or HA-GLT-1 7KR together with Nedd4-2-YFP (closed bars) or pcDNA3 (controls; open bar) and were assayed for [3H]glutamate uptake as in Fig. 4. The histograms represent the mean ± S.E. (error bars) of three replicate experiments, and they represent the percentage of the controls. *, statistically significantly different (p < 0.05; Student’s t test). IP, immunoprecipitation.
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FIGURE 6. Interaction between GLT-1 and Nedd4-2. A, COS-7 cells were transiently transfected with the constructs indicated, and after metabolic labeling (as indicated in Fig. 1), the cells were maintained in the presence (+) or absence of PMA (−; 0.5 μM) for 30 min. The cells were lysed, and Nedd4-2 was immunoprecipitated with anti-YFP antiserum. Immunoprecipitated material and lysates were processed as in A, probed with anti-HA, and reprobed with anti-YFP. IP, immunoprecipitation.

B

FIGURE 7. Effect of PMA on Nedd4-2 phosphorylation. COS-7 cells were transiently transfected with the constructs indicated, and after metabolic labeling (as indicated in Fig. 1), the cells were maintained in the presence (+) or absence of PMA (−; 0.5 μM) for 30 min. The cells were lysed, and Nedd4-2 was immunoprecipitated with anti-YFP antiserum. The proteins recovered were resolved by 10% SDS-PAGE, dried, and subjected to autoradiography. Gels were rehydrated in 50 mM ammonium bicarbonate, and the proteins were transferred onto a PVDF membrane that was probed with an anti-YFP antiserum. Three independent experiments were performed with similar results. IP, immunoprecipitation; WB, Western blot.

DISCUSSION

The crucial role of GLT-1 in shaping normal glutamatergic neurotransmission is reflected in numerous studies as is the involvement of GLT-1 in the pathophysiology of brain diseases that include both neurodegenerative processes and acute episodes (38, 39). Hence, regulating GLT-1 is an important aspect in the physiology and pathology of the nervous system, which is in part influenced by the trafficking of GLT-1 to and from the plasma membrane (3). PKC activation is known to participate in GLT-1 trafficking, increasing the rate of GLT-1 endocytosis from the cell surface to intracellular compartments, although how this process occurs is still not fully understood. Here, we demonstrate that although this transporter is phosphorylated in response to PKC activation this modification is not required for its internalization. Although PKC activation by phorbol esters increases the incorporation of 32P into GLT-1, a mutation at serine 520 abolishes this increase but does not affect its internalization after PMA treatment. Earlier studies failed to identify the target of phosphorylation and produced contradictory results (6, 36). Thus, based on chimeras constructed between GLT-1 and EAAC1, a region between residues 475 and 517 of the rat GLT-1 was proposed to be involved in PKC-dependent endocytosis (6). Mutation of the five serines in this 43-amino acid segment to alanines as well as the single mutant S486A attenuated the PKC-dependent loss of cell surface expression, although GLT-1 phosphorylation was only partially diminished (for S5SA), or it remained unaffected (for S486A) (6).

We hypothesize that the crucial role of the 43-residue segment is due to the presence of lysine 517, which has been shown to be involved in PKC-mediated ubiquitination (11). Moreover, Ser486 has previously been proposed to mediate PKC-dependent endocytosis, but it only marginally contributes to endocytosis in MDCK and COS cells and primary astrocytes (data not shown). Similarly, despite the dramatic decrease in phosphorylation of the S520A mutant induced by phorbol esters, this transporter is endocytosed like the wild type GLT-1. The dissociation of GLT-1 phosphorylation from endocytosis is reminiscent of that of DAT in which eliminating the C-terminal serines blocks PMA-induced phosphorylation but not its internalization (15). Furthermore, DAT internalization depends on its ubiquitination induced by phorbol esters (40, 41). Thus, the physiological role of GLT-1 phosphorylation remains unclear and might be related to the subsequent sorting of the transporter in the endosomal compartment.

As reported previously, a necessary step in the endocytosis of GLT-1 promoted by phorbol esters is its ubiquitination (11, 16). Here, we show that the ubiquitin ligase Nedd4-2 mediates PMA-induced ubiquitination of GLT-1, consistent with the demonstration in Xenopus oocytes that this enzyme regulates the activity of EAAT2, the human orthologue of GLT-1 (20). We show that the presence of Nedd4-2 increases the amount of ubiquitin incorporated into GLT-1, and it affects the stability of GLT-1 in mammalian cells. This is similar to another target

8C). Accordingly, the increase in ubiquitination promoted by PMA was lost in cells depleted of Nedd4-2 (Fig. 8D). In summary, these data support the involvement of Nedd4-2 in the down-regulation of GLT-1 promoted by the activation of PKC.
of Nedd4-2, the epithelial sodium channel (ENaC), that is ubiquitinlated by this ligase and then sorted into lysosomes (17).

Notably, our results indicate that phorbol esters promote the physical association of GLT-1 with Nedd4-2, whereas knockdown of endogenous Nedd4-2 in COS cells abolishes the effect of PMA on the ubiquitination and endocytosis of GLT-1. It is known that the WW domains of Nedd4-2 are involved in its binding to target proteins that display PY structural motifs (28). However, GLT-1 does not bear such a motif, suggesting the need for intermediate proteins to mediate the interaction or that they themselves are ubiquitinated by Nedd4-2. Nevertheless, the decrease in the ubiquitin that associates with the GLT-1 7KR mutant suggests that the transporter itself is directly ubiquitinlated by Nedd4-2, although additional components of the endocytic machinery might also be modified by Nedd4-2. The inhibitory effect of Nedd4-2 on EAAT2 was shown to be abrogated by SGK1 (20). Thus, the interaction of GLT-1 with Nedd4-2 is likely to be dynamic and regulated by diverse kinases. Nedd4-2 is indeed phosphorylated following the activation of several kinases, promoting either its association with or dissociation from target proteins. Indeed, SGK binds to and phosphorylates Nedd4-2, decreasing its affinity to its best characterized target, ENaC (42–44). Protein kinase A also phosphorylates Nedd4-2 at sites overlapping those modified by SGK (Ser-221, Ser-327, and Thr-246), and it inhibits Nedd4-2 binding to ENaC, although none of these residues are phosphorylated by PKC.3 Other kinases like Iα/H9260B kinase-α/H9252 and Akt1 also phosphorylate Nedd4-2 and inhibit the binding of Nedd4-2 to ENaC through a mechanism that recruits the 14-3-3 protein to the phosphorylated Nedd4-2, thereby precluding its binding to its target (45). By contrast, AMP-activated protein kinase phosphorylates Nedd4-2 and favors its binding to ENaC (46). Similarly, we observed an increase in the affinity of phosphorylated Nedd4-2 for GLT-1, although the exact role of phosphorylation in this association and whether

3 N. García-Tardón, J. Martínez-Villarreal, E. Fernández-Sánchez, C. Giménez, and F. Zafra, unpublished results.

FIGURE 8. Effect of Nedd4-2 knockdown on GLT-1 endocytosis. A, COS-7 cells were nucleofected with Nedd4-2 siRNA (+ siRNA), and after 24 hours in culture, the cells were transfected with HA-GLT-1. The next day, cells were exposed to PMA (1 μM; 30 min) or to the vehicle alone and then transferred to 4 °C. The membrane proteins were biotinylated, isolated with streptavidin-agarose beads, and analyzed in Western blots probed with an anti-HA antibody (WB: α-HA). The cytoplasmic protein calnexin was used as a biotinylation negative control (WB: α-CNX). Lysates were also analyzed in parallel and probed with anti-HA, anti-Nedd4-2 (WB: α-Nedd), and anti-calnexin antibodies as expression and loading controls. B and C, densitometric analysis of the average values of three independent Western blots like those presented in A for Nedd4-2 expression (B) and membrane biotinylated protein (C). The histograms represent the mean ± S.E. (error bars) of three triplicate experiments, and they represent the percentage of the controls. *, statistically significantly different (p < 0.05; Student’s t test). D, cells were nucleofected with Nedd4-2 siRNA and transfected with HA-GLT-1 as in A. [3H]Glutamate uptake was measured in cells pretreated with PMA (closed bars) or with vehicle (open bar). The data represent the mean ± S.E. (error bars) of three triplicate experiments, and they represent the percentage of the control activity, which was 12.5 ± 0.7 nmol of glutamate/mg of protein/10 min for HA-GLT-1 in the absence of siRNA. The values in the presence of PMA were compared with the values obtained with the vehicle alone (*, p < 0.01; Student’s t test). E, cells were nucleofected, transfected, and treated with PMA or vehicle as in A. Then cells were lysed, and the ubiquitinated proteins were isolated with agarose-conjugated anti-mult ubiquitin (IP: α-UB). The proteins recovered were analyzed in Western blots probed with anti-HA antibody (WB: α-HA). Lysates were processed in parallel and probed as in A.
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such increased phosphorylation is directly caused by PKC or additional kinases activated downstream of PKC remain unknown. Nevertheless, Nedd4-2 appears to be a central element in the regulatory cascade initiated by PKC, promoting the endocytosis and subsequent degradation of important transporters like DAT, GLT1, the cationic amino acid transporter, and perhaps other membrane proteins.

Ubiquitination of membrane proteins is commonly associated with the clathrin-dependent endocytic machinery (47). Indeed, GLT-1 endocytosis in response to phorbol esters has been reported to depend on the clathrin pathway (10, 12), and it is followed by the lysosomal degradation of the endocytosed transporter (12). However, the phorbol ester-promoted endocytosis of EAAT2 was recently shown to be dependent on flotillin-1 (13). We observed that siRNA-mediated depletion of flotillin-1 in MDCK and COS cells also inhibits the endocytosis of this transporter,3 raising the questions of whether ubiquitination might also regulate endocytosis mediated by flotillin-1-containing vesicles and whether clathrin- and flotillin-mediated endocytosis of GLT-1 coexists in certain cell types or physiological circumstances. In summary, our results support the involvement of Nedd4-2 in the PKC-regulated trafficking of one of the most abundant proteins of the CNS, the GLT-1 transporter, which plays important roles in the physiology and pathology of the nervous system.

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