Journal of Neurochemistry
Lippincott–Raven Publishers, Philadelphia

Inhibition of the High-Affinity Brain Glutamate Transporter GLAST-1 via Direct Phosphorylation

Marcus Conradt and Wilhelm Stoffel

Institute of Biochemistry I, Medical Faculty, University of Cologne, Cologne, Germany

Abstract: Neurotransmission at excitatory glutamatergic synapses is terminated by the reuptake of the neurotransmitter by high-affinity transporters, which keep the extracellular glutamate concentration below excitotoxic levels. The amino acid sequence of the recently isolated and cloned brain-specific glutamate/aspartate transporter (GLAST-1) of the rat reveals three consensus sequences of putative phosphorylation sites for protein kinase C (PKC). The PKC activator phorbol 12-myristate 13-acetate (PMA) decreased glutamate transport activity in *Xenopus* oocytes and human embryonic kidney cells (HEK293) expressing the cloned GLAST-1 cDNA, within 20 min, to 25% of the initial transport activity. This down-regulation was blocked by the PKC inhibitor staurosporine. GLAST-1 transport activity remains unimpaired by phorbol 12-monomyristate. Removal of all putative PKC sites of wild-type GLAST-1 by site-directed mutagenesis did not abolish inhibition of glutamate transport. [^{32}P]Phosphate-labeled wild-type and mutant transport proteins devoid of all predicted PKC sites were detected by immunoprecipitation after stimulation with PMA. Immunoprecipitation of [^{35}S]methionine-labeled transporter molecules indicates a similar stability of phosphorylated and nonphosphorylated GLAST-1 protein. Immunofluorescence staining did not differentiate surface staining of HEK293 cells expressing GLAST-1 with and without PMA treatment. These data suggest that the neurotransmitter transporter activity of GLAST-1 is inhibited by phosphorylation at a non-PKC consensus site. **Key Words:** Glutamate transporter—Protein kinase C—Phorbol ester—Phosphorylation.
J. Neurochem. **68,** 1244–1251 (1997).

Excitatory amino acids glutamate and aspartate are transmitters used by most fast excitatory synapses in the vertebrate CNS (Collingridge and Lester, 1989). However, the prolonged action of high extracellular concentrations of glutamate is excitotoxic (Choi et al., 1987). Sodium-dependent, high-affinity uptake systems of glutamate into nerve terminals and glial cells terminate glutamatergic neurotransmission and prevent glutamate toxicity (Rosenberg et al., 1992; Amara and Kuhar, 1993).

The recently cloned Na^+-dependent glutamate transporters (GLAST-1, GLT-1, EAAC1, and EAAT4) form a new family of integral membrane proteins with ~50% amino acid identities (Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992; Fairman et al., 1995). The human homologous proteins exhibiting amino acid identities of 92–96% to both the rat and the rabbit sequences have been described recently (Arriza et al., 1994). Hydropathy plots of the transporters suggest the existence of six putative transmembrane α-helices bearing two experimental verified glycosylation sites between transmembrane helix 3 and 4 (Conradt et al., 1995). The sequence homology of the transporters is strongest at the C-terminus, suggesting its possible participation in the transport process. This assumption has been confirmed by the identification of three amino acid residues in the C-terminal part of the protein that are essential for glutamate transport activity (Conradt and Stoffel, 1995; Pines et al., 1995).

Immunological studies have localized GLAST-1 and GLT-1 in astroglial cells, whereas EAAC1 is a neuronal transporter that is also expressed in kidney and intestine (Lehre et al., 1995). The cellular specificity of EAAT4 expression has not yet been determined. The two astroglial transporters GLAST-1 and GLT-1 are expressed throughout the brain at different levels in different regions. Immunocytochemistry revealed that GLAST-1 is strongly expressed in the cerebellum and also in the hippocampal formation. GLT-1, on the other hand, exhibits a high immunoreactivity in the hippocampus, but a less intensive immunoreactivity in the cerebellum (Chaudhry et al., 1995).

Received July 15, 1996; revised manuscript received October 15, 1996; accepted October 15, 1996.

Address correspondence and reprint requests to Dr. W. Stoffel at Institute of Biochemistry I, Medical Faculty, University of Cologne, Joseph-Stelzmann-Str. 52, D-50931 Cologne, Germany.

Abbreviations used: BS, Barth's modified solution; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; ECS, extracellular solution; GABA, γ-aminobutyric acid; GLAST-1, glutamate/aspartate transporter; HEK, human embryonic kidney; $I_{GLAST-1}$, GLAST-1–mediated inward currents; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PMM, phorbol 12-monomyristate; SDS, sodium dodecyl sulfate; SOE, splicing by overlapping extension; T, triple mutant S116A, T341A, T372A.

The existence of multiple, differentially distributed glutamate transporters suggests that these plasma membrane proteins may function not merely by clearing the synaptic cleft from excitatory amino acids. They may modulate excitation by rapidly buffering the neurotransmitter concentration during transmission as well as by regulating tonic ambient glutamate levels (Barbour et al., 1994; Mennerick and Zorumski, 1994; Tong and Jahr, 1994). An essential step toward understanding the contribution of neurotransmitter transporters to the modulation of synaptic transmission is the analysis of the regulation of the transporter. Previous studies have shown that γ-aminobutyric acid (GABA) transport is activated by protein kinase C (PKC) stimulated by phorbol esters (Corey et al., 1994), whereas dopamine and glycine transport into cells expressing the respective recombinant transporters is reduced by phorbol 12-myristate 13-acetate (PMA)-mediated activation of protein kinase (Kitayama et al., 1994; Sato et al., 1995). Using primary cultures from rat brain cerebral cortex, Casado et al. (1991) reported that phorbol esters produced a 50% increase in glial glutamate uptake, suggesting that phosphorylation is of physiological relevance. Accordingly, it has been shown that phorbol ester–mediated activation of PKC results in stimulation of expressed glial glutamate transporter GLT-1 (Casado et al., 1993). As mentioned above, GLT-1 represents the predominant glial transporter in the cerebrum, whereas GLAST-1 is highly enriched in the cerebellum. The present study revealed that GLAST-1 is inhibited by PMA, known to induce the activation of PKC. Thus, phosphorylation exerts an opposite effect on the function of differentially expressed glial glutamate transporters GLAST-1 and GLT-1. Elimination of the predicted PKC sites of GLAST-1 followed by in vivo [^{32}P]phosphate labeling clearly showed that GLAST-1 is phosphorylated at a non-PKC consensus site. Neither the stability nor the subcellular distribution of the transporter appears to be affected by phosphorylation. Thus, the decrease in glutamate transport activity is most probably evoked by direct phosphorylation of GLAST-1, leading to an inactivation of the transporter.

MATERIALS AND METHODS

Materials

T4 DNA ligase, SP6 polymerase, and restriction enzymes were obtained from GibcoBRL or Boehringer Mannheim. PMA, phorbol 12-monomyristate (PMM), dimethylamiloride, staurosporine, Cy3-conjugated streptavidin, monoclonal anti-α-tubulin mouse IgG, fluorescein isothiocyanate–labeled goat anti-rabbit IgG, and protein A–Sepharose CL-4B were purchased from Sigma. [^{32}P]Orthophosphate and [^{35}S]methionine were from Amersham. The voltage-clamp amplifier of Warner Instruments Corp. and the hardware and software package ISO2 from MFK (Frankfurt) were used in the whole-cell voltage-clamp experiments.

Site-specific mutagenesis

The plasmid pSP-GLAST (Storck et al., 1992) served as template in splicing by overlapping extension (SOE) PCR (Higuchi et al., 1988). Three mutant oligonucleotides and their antisense primers replaced the wild-type codons, as follows: Ser^{116} by Ala (S116A), 5′-TAAGGCAGCTGG-GAAGATGG-3′; Thr^{341} by Ala (T341A), 5′-CCTGGT-AGCCCGGAAGAACC-3′; and Thr^{372} by Ala (T372A), 5′-GCCCATCGCTTTCAAGTGCCT-3′. The oligonucleotide sequences represent the sense strand. The three mutated cDNA fragments resulting from the SOE reaction were subcloned into pSP-GLAST. The nucleotide sequences of the subcloned DNA fragments between the used restriction sites were determined. The triple mutant S116A, T341A, T372A (T) was constructed by an additional SOE-PCR and standard cloning procedures using the synthesized mutant constructs bearing the respective point mutations. The cDNA of the triple mutant T was subcloned as *Not*I fragment into the mammalian expression vector pRC/CMV (Invitrogen). The wild-type GLAST-1 cDNA subcloned into the pRC/CMV vector has been described previously (Stoffel and Blau, 1995).

Expression in oocytes and cell transfection

Wild-type and mutant pSP-GLAST cDNA were linearized with *Eco*RI. The respective cRNAs were synthesized from their corresponding templates using SP6 RNA polymerase. Stage V–VI oocytes were defolliculated with collagenase and injected with 40 nl of cRNA (0.5 μg/μl). cRNA preparation and injection of the oocytes have been described previously (Storck et al., 1992). Human embryonic kidney cells (HEK293 cells, American Type Culture Collection CRL 1573) stably expressing wild-type GLAST-1 or the triple mutant T were generated by electroporation as described previously (Stoffel and Blau, 1995).

Electrophysiology

Electrogenic transport in oocytes was assayed by voltage-clamp, using the two-electrode voltage-clamp amplifier. In brief, oocytes were voltage clamped at −90 mV and superfused continuously with Barth's modified saline (BS). The agents were applied by bath application at the indicated concentration in BS. Microelectrodes filled with 3 *M* KCl had a resistance range of 1–2 MΩ.

Recordings from HEK293 cells were made during superfusion with an extracellular solution (ECS) containing 150 m*M* NaCl, 3 m*M* KCl, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 10 m*M* glucose, and 10 m*M* HEPES, pH 7.4. Whole-cell recordings were performed with patch pipettes (3–7 MΩ) filled with 140 m*M* KCl, 2 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 10 m*M* EGTA, 2 m*M* ATP, and 10 m*M* HEPES, pH 7.2. The HEK293 cells had resting potentials of −40 to −65 mV. Drugs and ECS (50–100 μ*M*) were applied to HEK293 cells with a rapid perfusion system consisting of a multibarreled electrode that was positioned within a distance of 50 to 100 μm to the cell. Voltage-clamp currents were recorded with an Axopatch 200A amplifier (Axon Instruments), digitized, and acquired on a computer (Mac II CI) using an ITC-16 interface (Instrutech Corp.) and the Pulse software package from HEKA (Germany).

The data of the concentration–response curves were fitted by nonlinear regression to minimize the squared errors to the Hill equation, $I = I_{max} \times [S]^n/([S]^n + K_m^n)$, where I_{max} is the normalized maximal current, $[S]$ the substrate concentration, K_m the apparent affinity constant, and n the cooperativity parameter. Data were expressed as mean ± SD values.

In vivo labeling and immunoprecipitation

HEK293 cells (10^6) were metabolically labeled with [^{32}P]orthophosphate (250 μCi/ml) in phosphate-free Dulbecco's modified Eagle medium (DMEM) for 2 h or with [^{35}S]methionine (100 μCi/ml) in methionine-deficient DMEM for 24 h. After treatment of cells with 250 n*M* PMA in DMEM for 0.5 h they were washed three times in ice-cold phosphate-buffered saline [PBS; 6.5 m*M* Na_2HPO_4, 1.45 m*M* KH_2PO_4 (pH 7.2), 136 m*M* NaCl, 2.68 m*M* KCl] and lysed in "immunoprecipitation buffer" (1 m*M* Na_3VO_4, 10 m*M* Na_2HPO_4, 1% Triton, 20 m*M* NaF, 50 m*M* NaCl, and 20 m*M* Tris, pH 7.6). Lysates were centrifuged at 12,000 *g* for 5 min at 4°C. Supernatants were incubated at 4°C for 2.5 h with anti-GLAST antibody (Storck et al., 1992). Antigen–antibody complex was bound by protein A–Sepharose at 4°C for 2 h. After immunoprecipitation, beads were washed five times in immunoprecipitation buffer supplemented with 0.1% sodium dodecyl sulfate (SDS) and three times with 10 m*M* Tris-HCl, pH 7.6. Precipitated proteins were dissociated and reduced by incubating the samples dissolved in Laemmli sample buffer (0.0625 *M* Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol, and 0.1% bromphenol blue) for 2 min at 95°C. Proteins were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and visualized by phosphorimaging with a Phosphorimager 445 SI (Molecular Dynamics).

Immunohistochemistry

HEK293 cells expressing GLAST-1 were grown on poly-D-lysine–coated coverslips. At subconfluence, cells were processed for immunostaining. The agents were added to the growth medium of the cell cultures at times indicated before they were processed further. Cells were washed twice with ice-cold PBS and fixed at room temperature with 1% paraformaldehyde in PBS for 10 min. After fixation, the cells were washed three times with PBS for five min and blocked with PBS containing 10% nonimmune serum, 4% bovine serum albumin (BSA) at 4°C for 1 h. Cells were incubated for 1.5 h at 4°C with a biotinylated polyclonal anti-GLAST antibody in 1% BSA/PBS, washed five times with PBS for 5 min, and incubated for 30 min at room temperature with Cy3-conjugated streptavidin. Cells were washed five times with PBS for 10 min, sealed with coverslips, examined with a Zeiss Axioscope fluorescence microscope, and photographed with a Zeiss MC 100 microscope camera.

Immunostaining of α-tubulin was performed after permeabilization of cells with 0.25% Triton for 5 min at room temperature. Permeabilized cells were stained as described above using a monoclonal anti-α-tubulin IgG and a fluorescein 5′-isothiocyanate–conjugated secondary antibody.

RESULTS

PMA application decreased GLAST-1–mediated glutamate transport activity

In vitro transcribed wild-type GLAST-1 cRNA was microinjected in *Xenopus* oocytes and its expression verified by immunoprecipitation of the [^{35}S]methionine-labeled proteins. Anti–GLAST-1 antibodies immunoprecipitate a protein with an apparent molecular mass of 60–65 kDa in SDS-polyacrylamide gels (Fig. 1, lane 2) corresponding to the molecular mass of the purified transporter from rat brain (Schulte and Stoffel, 1995). The strong signal at approximately twice the molecular mass represents the dimeric form of the transporter (Conradt et al., 1995). Water-injected control oocytes expressed no immunoprecipitable protein (lane 1).

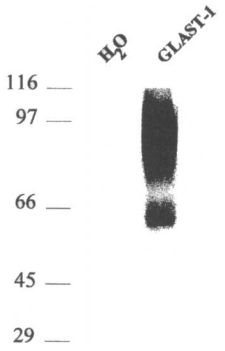

FIG. 1. Immunoprecipitations of wild-type GLAST-1 protein expressed in *Xenopus* oocytes. GLAST-1 cRNA was injected into *Xenopus* oocytes and incubated in Barth's medium supplemented with [^{35}S]methionine. Oocytes were homogenized and immunoprecipitated as described in Materials and Methods. Samples were analyzed by SDS-PAGE and phosphorimaging. Sizes of marker proteins are in kilodaltons. Lane 1, water-injected control oocytes; lane 2, oocytes injected with GLAST-1 cRNA.

We have shown previously that glutamate transport is electrogenic with a proposed stoichiometry of three Na^+ ions cotransported per molecule of glutamate, accompanied by the countertransport of one K^+ ion (Klöckner et al., 1993, 1994). Therefore, we used the whole-cell voltage-clamp technique to analyze glutamate uptake in oocytes expressing GLAST-1 and to investigate the influence of PMA on glutamate transport. The GLAST-1–mediated inward currents ($I_{GLAST-1}$), evoked by application of 100 μ*M* L-glutamate (saturation concentration) at different times after exposing the oocytes to PMA, were measured. Figure 2A indicates that the glutamate transport activity of GLAST-1 is reduced in a time- and dose-dependent manner. The velocity of the glutamate transport declines more rapidly at elevated PMA concentrations to reach a lower plateau compared with the application of PMA at lower concentration. Once the plateau is reached, even 1 m*M* L-glutamate concentrations do not evoke elevated currents, which suggests that V_{max} and not the K_m value of the transporter is reduced. PMM, a phorbol ester that does not activate PKC, has no effect on glutamate uptake at a concentration of 350 n*M* and an exposure time of 1 h (data not shown).

To exclude the possibility that the PMA-induced decrease in glutamate transport activity was due to a diminished Na^+ gradient evoked by the activation of Na^+/H^+ exchange, which is stimulated by PMA in some cell types, the PMA-mediated effect was studied in the presence of the Na^+/H^+-exchange inhibitor dimethylamiloride (Besterman et al., 1985). This drug (used at a concentration of 10 μ*M*) did not alter the PMA-induced decrease in glutamate transport activity in oocytes expressing GLAST-1 (data not shown).

The effect of staurosporine, an inhibitor of PKC, on the PMA-mediated reduction of glutamate transport was examined by simultaneous application of PMA and staurosporine at concentrations of 250 n*M* and 10 μ*M*, respectively. Preincubation of oocytes expressing GLAST-1 with 10 μ*M* staurosporine does not by itself affect $I_{GLAST-1}$. However, it strongly diminishes the in-

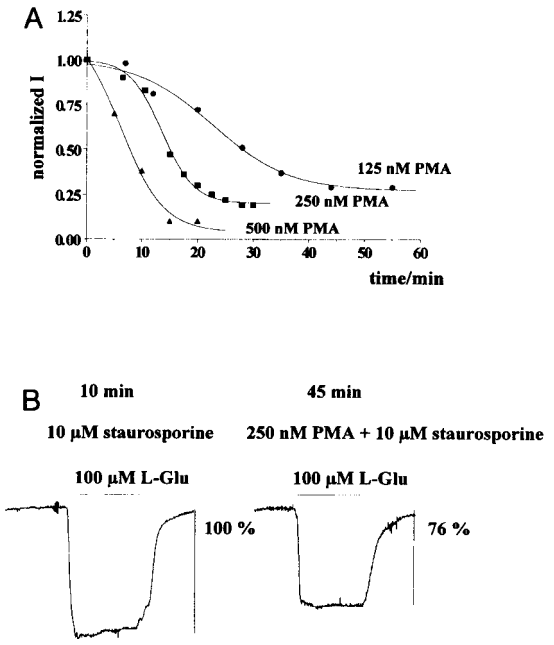

FIG. 2. Characterization of the PMA-induced decrease in GLAST-1–mediated glutamate transport. **A:** GLAST-1 activity is modulated in a time- and dose-dependent manner by PMA. GLAST-1–mediated inward currents ($I_{GLAST-1}$) evoked by application of 100 μM L-glutamate were measured at different times after bath application of 125, 250, and 500 nM PMA. The holding potential was −90 mV (90 mM $[Na^+]_o$). The current of each oocyte was normalized to the current amplitude before PMA application. Data shown represent the values of a single representative experiment performed in triplicate with individual determinations deviating from the mean by <15%. **B:** The PMA-mediated effect is strongly reduced by the PKC inhibitor staurosporine. The original traces were recorded from GLAST-1–expressing oocytes after the indicated incubation steps. A preincubation step of 10 min with 10 μM staurosporine was followed by superfusion with PMA and staurosporine for 45 min at concentrations of 250 nM and 10 μM, respectively. Preincubation of oocytes with 10 μM staurosporine does not by itself affect $I_{GLAST-1}$. The reduction of $I_{GLAST-1}$ after 45 min was only 25 ± 7% (n = 4) compared with 80 ± 11% (n = 3) without staurosporine. Data indicated are mean ± SD values obtained from three or four different oocytes.

duced decrease of GLAST-1–mediated currents in the presence of PMA. Figure 2B shows that even 45 min after coapplication of PMA/staurosporine, the reduction of $I_{GLAST-1}$ was only 25% compared with ~80% reduction without staurosporine (Fig. 2A).

Elimination of putative PKC sites has no influence on sensitivity of GLAST-1–mediated glutamate transport to PMA

We used site-directed mutagenesis to generate mutant GLAST-1 transporters, in which the predicted PKC sites were eliminated separately (S116A, T341A, and T372A) and together resulting in the triple mutant (T) lacking all PKC consensus sites. The impact of the point mutations on the functional properties of the glutamate transporter was analyzed electrophysiologically in the oocyte-expression system. The apparent K_m values of wild-type and mutant GLAST-1 were determined and showed no significant differences compared with the wild type (Fig. 3A).

The PMA sensitivity of mutant GLAST-1 was analyzed as described above. The removal of the PKC consensus sites did not affect the modulation of the transporter by PMA compared with the wild type (Fig. 3B). Similar results were obtained from four different experiments. We conclude that the modulation results from direct phosphorylation of a PKC nonconsensus site or is due to an indirect effect.

PMA treatment did not alter cell surface location of GLAST-1

It has been shown recently that PMA-induced activation of PKC is involved in the regulation of neurotransmitter transporter and receptor activity via movement of the integral membrane proteins to and from the plasma membrane (Corey et al., 1994; Ehlers et al., 1995). We addressed whether the PMA-mediated

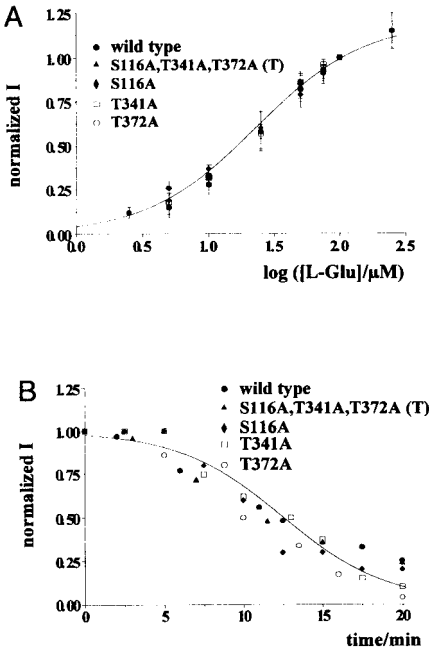

FIG. 3. Comparison of L-glutamate affinities and PMA sensitivities of wild-type and mutant GLAST-1. **A:** L-Glutamate K_m values. Data are mean values, bars the standard deviation of $I_{GLAST-1}$ (n = 2–11). The current of each oocyte was normalized to the current amplitude at 100 μM L-glutamate. The solid line is fitted to the data of wild-type GLAST-1 by minimizing squared errors according to the equation, $I = I_{max} \times [S]^n/([S]^n + K_m^n)$, with an apparent K_m value of 21 ± 3 μM L-glutamate (n = 11). The affinity for L-glutamate of the mutant transporters S116A, T341A, T372A, and T showed no significant differences and had apparent K_m values of 20 ± 3.5 (n = 3), 20 ± 1 (n = 2), 18 ± 3.5 (n = 3), and 21 ± 4 μM L-glutamate (n = 5), respectively. **B:** Analysis of PMA response of mutant transporters as described in Fig. 2A, except that the PMA concentration was 250 nM. There were no significant differences in the PMA sensitivity of wild-type and mutant transporters. The solid line is fitted to the data of wild-type GLAST-1.

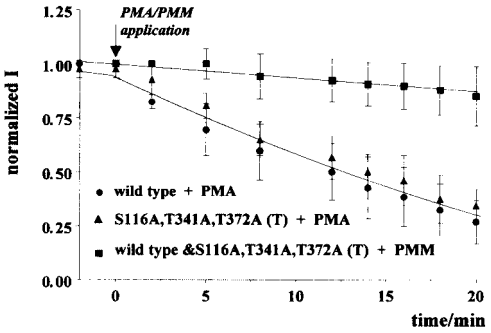

FIG. 4. Effect of PKC activation on transport of GLAST-1 expressed in HEK293 cells. The PMA response of wild-type and triple mutant T stably expressed in HEK293 cells was determined as described in Fig. 2A. The wild-type– and mutant T–mediated currents decreased within 20 min to ~25% of the initial value, which is comparable with the reduction observed in the *Xenopus* oocyte system. PMM leaves the transport activity of wild-type and triple mutant T unimpaired. Data shown represent mean ± SD values from four to six cells.

decrease in GLAST-1 activity might be due to internalization of the transporter by immunocytochemical studies on a HEK293 cell line stably transfected with GLAST-1.

First, we ascertained that the response of GLAST-1 on PMA treatment was not altered by the change of the heterologous expression system. The PMA sensitivity of wild-type transporter and the mutant T were analyzed by the voltage-clamp technique as described above. Figure 4 shows that PMA treatment of HEK293 cells expressing wild-type or the mutant protein T results in a decrease of GLAST-1–evoked currents that is comparable with the effect observed in the oocyte-expression system. PMM has no influence on the transporter activity. Thus, the PKC-induced modulation of GLAST-1 is independent of the expression system.

To exclude the possibility that PMA permeabilized the cell membrane enabling the antibody to recognize intracellularly localized GLAST-1, we performed immunostaining with an anti–α-tubulin antibody with permeabilized and nonpermeabilized cells after PMA treatment. Permeabilized cells (Fig. 5B) yielded an immunopositive reaction when the ubiquitous expression and intracellular localization of α-tubulin were probed, whereas no fluorescence was detected in nonpermeabilized cells (Fig. 5A). Thus, PMA treatment does not permeabilize the cell membrane.

The surface location of GLAST-1 was examined before and after incubation of GLAST-1–expressing HEK293 cells with 250 n*M* PMA for 45 min (Fig. 5C and D). No differences in the intensity of fluorescence were observed after immunostaining with an anti–GLAST-1–specific antibody, suggesting that the membrane targeting of the transporter was not affected by PMA treatment. Nontransfected HEK293 cells exhibit no immunofluorescence (data not shown). These data suggest that the membrane targeting of the transporter is not altered by PMA-mediated modulation of GLAST-1.

In vivo labeling of GLAST-1 with [^{32}P]phosphate after stimulation with PMA

Although the elimination of all predicted PKC sites did not abolish the effect of PMA treatment, we cannot exclude the possibility that PKC phosphorylates GLAST-1 at a PKC-like nonconsensus site. Therefore, we incubated wild-type– and triple mutant T–express-

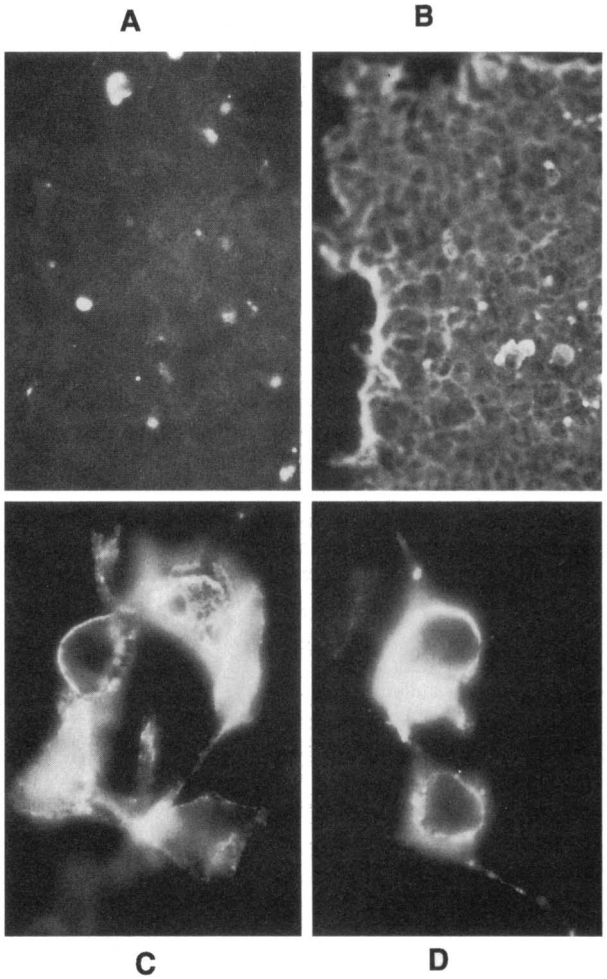

FIG. 5. A and **B:** Effect of PMA treatment on membrane permeability and subcellular distribution of GLAST-1. GLAST-1–expressing HEK293 cells were incubated with an anti–α-tubulin antibody and stained with fluorescein-conjugated secondary antibody after PMA treatment for 45 min. No fluorescence was detected in nonpermeabilized cells (A), whereas all permeabilized cells (B) react immunopositively. The few fluorescent cells in A are most probably mechanically disrupted cells. **C** and **D:** Surface location of GLAST-1 after PMA-mediated activation of PKC. HEK293 cells expressing the glutamate transporter were labeled with a biotinylated anti–GLAST-1 antibody before (C) and after (D) incubation with 250 n*M* PMA for 45 min. Immunofluorescence detection was achieved with streptavidin-coupled Cy3. No differences in the intensity of surface fluorescence were observed. Nontransfected HEK293 cells exhibit no immunofluorescence (data not shown).

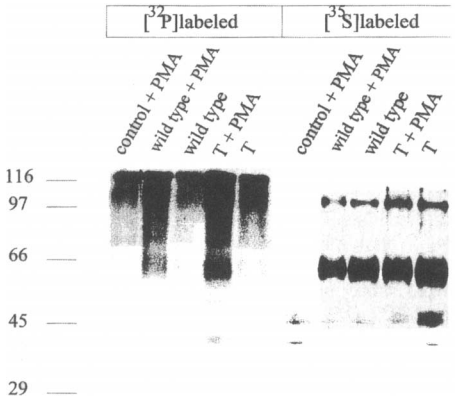

FIG. 6. Immunoprecipitations of [^{32}P]phosphate- and [^{35}S]-methionine-labeled wild-type and triple mutant T phosphoproteins. HEK293 cells were metabolically labeled with [^{32}P]-orthophosphate (lanes 1–5; lane numbers are counted from left to right) or with [^{35}S]methionine (lanes 6–10). Cells were lysed before (lanes 3, 5, 8, and 10) and after (lanes 1, 2, 4, 6, 7, and 9) activation with 250 n*M* PMA (0.5 h) and subjected to immunoprecipitation. Samples were analyzed by SDS-PAGE and phosphorimaging. Sizes of marker proteins are indicated in kilodaltons. Activation of PKC strongly increased [^{32}P]phosphate labeling of wild-type GLAST-1 and mutant T (lanes 2 and 4), whereas immunoprecipitations of non–PMA-treated cells yielded only weak signals of [^{32}P]phosphate-labeled GLAST-1 (lanes 3 and 5). Comparable amounts of [^{35}S]methionine-labeled proteins were immunoprecipitated before and after PMA treatment. In nontransfected control cells, no protein with a molecular mass corresponding to the 65 kDa of GLAST-1 was detectable on SDS-PAGE (lanes 1 and 6).

ing HEK293 cells for 2 h with [^{32}P]orthophosphate to label endogenous ATP. Cells were lysed before (lanes 3, 5, 8, and 10) and after stimulation of PKC (lanes 1, 2, 4, 6, 7, and 9) with 250 n*M* PMA for 0.5 h and subjected to immunoprecipitation as described in Materials and Methods. In nontransfected cells, no protein with a molecular mass corresponding to the 65 kDa of GLAST-1 was detectable on SDS-PAGE (Fig. 6, lanes 1 and 6). After PMA treatment of PKC, the phosphorylation state of wild-type GLAST-1 and the mutant T is strongly increased as indicated by the intensive band at 65 kDa (lanes 2 and 4). Cells that were not treated with PMA yielded only weak signals of [^{32}P]phosphate-labeled GLAST-1 (lanes 3 and 5). This clearly indicates that PMA activates a kinase that leads to a direct phosphorylation of GLAST-1 at a nonconsensus PKC site (lanes 4 and 5).

In a parallel experiment, transport proteins were metabolically labeled with [^{35}S]methionine to investigate the expression rate and stability of GLAST-1 in response to PMA. No significant differences in band intensities before and after activation were observed, which suggests that phosphorylation has no influence on the expression rate or stability of GLAST-1 (lanes 7–10).

DISCUSSION

Four related glutamate transporters have been cloned that are differentially expressed throughout the brain, suggesting that they participate in different ways in the regulation of excitatory amino acid concentration in the synaptic cleft (Lehre et al., 1995). The stimulation or inhibition of neurotransmitter transport via second-messenger pathways could play a significant role in synaptic function. In this study, we demonstrated that the glutamate transporter GLAST-1 expressed in *Xenopus* oocytes and HEK293 cells is modulated by PKC.

Treatment of GLAST-1–expressing cells with low doses of PMA, a tumor-promoter agent known to stimulate PKC (Nishizuka, 1986), decreased glutamate transport activity. PMA-induced inhibition could be blocked by the simultaneous application of the PKC inhibitor staurosporine. This indicates that PKC was responsible for the decrease in transport. However, elimination of all putative PKC consensus sites by site-directed mutagenesis sustained the PMA-mediated effect. In a similar manner, removal of all potential PKC consensus sites in the GABA transporter GAT1 had no influence on the PMA-induced increase of GABA transport activity, which was regulated by a subcellular redistribution of the transport protein (Corey et al., 1994). That GLAST-1 may be regulated via an altered targeting to the plasma membrane is ruled out by immunofluorescence studies indicating that the transporter remains localized in the plasma membrane after PMA treatment. We showed by immunoprecipitations of phosphorylated wild-type and mutant transporter lacking all putative PKC phosphorylation sites that phosphorylation occurred at a nonconsensus site. Because the degree of phosphorylation strongly increased in the presence of PMA with a progressive decrease in transport activity, we concluded that the inactivation of GLAST-1 is due to an enhanced phosphorylation of the transporter.

The related glutamate transporter GLT-1 is phosphorylated at a PKC consensus site (Ser^{113}), which results in a twofold increase of glutamate uptake (Casado et al., 1993). This PKC site (Ser^{116}) is also present in the GLAST-1 amino acid sequence but is obviously not involved in the regulation of GLAST-1, as proved by analysis of the mutants S116A and the triple mutant lacking all PKC sites. The phosphorylation of GLAST-1 leads to an inactivation of the transporter, in contrast to the stimulatory effect exerted by PMA on GLT-1. Therefore, we suggest that GLAST-1 and GLT-1 are phosphorylated at different sites, resulting in an opposite modulation of transport activity. PMA-induced glutamate uptake in primary rat brain cerebellar slices (Casado et al., 1991) is not in conflict with our data because it mainly reflects the activity of GLT-1, which is the predominant glial transporter in the cerebrum. Differential modulation of the corresponding human glutamate transporters EAAT1–3 by arachidonic acid supports that differences in the transporter response to second messengers might play an important role in synaptic function (Zerangue et al., 1995).

At the present time, we can only speculate about the physiological relevance of glutamate transporter modulation by PKC. In view of the growing evidence that glial cells express nearly all types of glutamate receptors including phospholipase C–coupled metabotropic glutamate receptors (Gallo and Russell, 1995), it is conceivable that glutamatergic transmission results in an activation of the phosphatidylinositol cycle in astrocytes (Pearce et al., 1986). This would activate PKC, leading to phosphorylation of GLAST-1, which exerts an inhibitory effect on the glutamate uptake. The elevated glutamate concentration might increase synaptic efficacy.

Signaling pathways more complex than direct modulation of GLAST-1 by PKC, such as phosphorylation via a PKC-activated tyrosine kinase, may be involved in the regulation of GLAST-1. It is well known that long-term potentiation in the hippocampus is blocked by tyrosine kinase inhibitors (O'Dell et al., 1991). The delayed rectifier-type potassium channel Kv 1.2 (Huang et al., 1993) is highly expressed in the CNS and rapidly phosphorylated by the action of tyrosine kinase PYK2 with the activation of the mitogen-activated protein kinase signaling pathway, for instance (Lev et al., 1995). Further investigations are required to study the regulatory role of tyrosine kinases in the activation of the electrogenic L-glutamate transporters.

Acknowledgment: The generous support of the Deutsche Forschungsgemeinschaft (SFB 243) and a predoctoral fellowship within the Graduierten-Kolleg (to M.C.) are gratefully acknowledged.

REFERENCES

Amara S. G. and Kuhar M. J. (1993) Neurotransmitter transporter: recent progress. *Annu. Rev. Neurosci.* **16,** 73–93.

Arriza J. L., Fairman W. A., Wadiche J. I., Murdoch G. H., Kavanaugh M. P., and Amara S. G. (1994) Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. *J. Neurosci.* **14,** 5559–5569.

Barbour B., Keller B. U., Llano I., and Marty A. (1994) Prolonged presence of glutamate during excitatory synaptic transmission to cerebellar Purkinje cells. *Neuron* **12,** 1331–1343.

Besterman J. M., May W. S. Jr., LeVine H., Cragoe E. J. Jr., and Cuatrecasas P. (1985) Amiloride inhibits phorbol ester-stimulated Na^+/H^+ exchange and protein kinase C. An amiloride analog selectively inhibits Na^+/H^+ exchange. *J. Biol. Chem.* **260,** 1155–1159.

Casado M., Zafra F., Aragón, C., and Giménez C. (1991) Activation of high-affinity uptake of glutamate by phorbol esters in primary glial cell cultures. *J. Neurochem.* **57,** 1185–1190.

Casado M., Bendahan A., Zafra F., Danbolt N. C., Aragón C., Giménez C., and Kanner B. I. (1993) Phosphorylation and modulation of brain glutamate transporters by protein kinase C. *J. Biol. Chem.* **268,** 27313–27317.

Chaudhry F. A., Lehre K. P., van Lookeren Campagne M., Ottersen O. P., Danbolt N. C., and Storm-Mathisen J. (1995) Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. *Neuron* **15,** 711–720.

Choi D. W., Maulucci-Gedde M., and Kriegstein A. R. (1987) Glutamate neurotoxicity in cortical cell culture. *J. Neurosci.* **7,** 357–368.

Collingridge G. L. and Lester R. A. J. (1989) Excitatory amino acid receptors in the vertebrate central nervous system. *Pharmacol. Rev.* **40,** 143–210.

Conradt M. and Stoffel W. (1995) Functional analysis of the high affinity, Na^+-dependent glutamate transporter GLAST-1 by site directed mutagenesis. *J. Biol. Chem.* **270,** 25207–25212.

Conradt M., Storck T., and Stoffel W. (1995) Localization of *N*-glycosylation sites and functional role of the carbohydrate units of GLAST-1, a cloned rat brain L-glutamate/L-aspartate transporter. *Eur. J. Biochem.* **229,** 682–687.

Corey J. L., Davidson N., Lester H. A., Brecha N., and Quick M. W. (1994) Protein kinase C modulates the activity of a cloned γ-aminobutyric acid transporter expressed in *Xenopus* oocytes via regulated subcellular redistribution of the transporter. *J. Biol. Chem.* **269,** 14759–14767.

Ehlers M. D. Tingley W. G., and Huganir R. L. (1995) Regulated subcellular distribution of the NR1 subunit of the NMDA receptor. *Science* **269,** 1734–1737.

Fairman W. A., Vandenberg R. J., Arriza J. L., Kavanaugh M. P., and Amara S. G. (1995) An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. *Nature* **375,** 599–603.

Gallo V. and Russell J. T. (1995) Excitatory amino acid receptors in glia: different subtypes for distinct functions. *J. Neurosci. Res.* **42,** 1–8.

Higuchi R., Krummel B., and Saiki R. K. (1988) A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res.* **16,** 7351–7367.

Huang X. Y., Morielli A. D., and Peralta E. G. (1993) Tyrosine kinase-dependent suppression of a potassium channel by the G protein-coupled m1 muscarinic acetylcholine receptor. *Cell* **75,** 1145–1156.

Kanai Y. and Hediger M. A. (1992) Primary structure and functional characterization of a high-affinity glutamate transporter. *Nature* **360,** 467–471.

Kitayama S., Dohi T., and Uhl G. R. (1994) Phorbol esters alter functions of the expressed dopamine transporter. *Eur. J. Pharmacol.* **268,** 115–119.

Klöckner U., Storck T., Conradt M., and Stoffel W. (1993) Electrogenic L-glutamate uptake in *Xenopus laevis* oocytes expressing a cloned rat brain L-glutamate/L-aspartate transporter (GLAST-1). *J. Biol. Chem* **268,** 14594–14596.

Klöckner U., Storck T., Conradt M., and Stoffel W. (1994) Functional properties and substrate specificity of the cloned L-glutamate/L-aspartate transporter GLAST-1 from rat brain expressed in *Xenopus* oocytes. *J. Neurosci.* **14,** 5759–5765.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lehre K. P., Levy L. M., Ottersen O. P., Storm-Mathisen J., and Danbolt N. C. (1995) Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. *J. Neurosci.* **15,** 1835–1853.

Lev S., Moreno H., Martinez R., Canoll P., Peles E., Musacchio J. M., Plowman G. D., and Schlessinger J. (1995) Protein tyrosine kinase PYK2 involved in Ca^{2+}-induced regulation of ion channel and MAP kinase functions. *Nature* **376,** 737–745.

Mennerick S. and Zorumski C. F. (1994) Glial contributions to excitatory neurotransmission in cultured hippocampal cells. *Nature* **368,** 59–62.

Nishizuka Y. (1986) Studies and perspectives of protein kinase C. *Science* **233,** 305–312.

O'Dell T. J., Kandel E. R., and Grant S. G. (1991) Long-term potentiation in the hippocampus is blocked by tyrosine kinase inhibitors. *Nature* **353,** 558–560.

Pearce B., Albrecht J., Morrow C., and Murphy S. (1986) Astrocyte glutamate receptor activation promotes inositol phospholipid turnover and calcium flux. *Neurosci. Lett.* **72,** 335–340.

Pines G., Danbolt N. C., Bjoras M., Zhang Y., Bendahan A., Eide L., Koepsell H., Storm-Mathisen J., Seeberg E., and Kanner B. I. (1992) Cloning and expression of a rat brain L-glutamate transporter. *Nature* **360,** 464–467.

Pines G., Zhang Y., and Kanner B. I. (1995) Glutamate 404 is involved in the substrate discrimination of GLT-1, a (Na^+ + K^+)-coupled glutamate transporter from rat brain. *J. Biol. Chem.* **270,** 17093–17097.

Rosenberg P. A., Amin S., and Leitner M. (1992) Glutamate uptake disguises neurotoxic potency of glutamate agonists in cerebral cortex in dissociated cell culture. *J. Neurosci.* **12,** 56–61.

Sato K., Adams R., Betz H., and Schloss P. (1995) Modulation of a recombinant glycine transporter (GLYT1b) by activation of protein kinase C. *J. Neurochem.* **65,** 1967–1973.

Schulte S. and Stoffel W. (1995) UDP galactose:ceramide galactosyltransferase and glutamate/aspartate transporter. Copurification, separation and characterization of the two glycoproteins. *Eur. J. Biochem.* **233,** 947–953.

Stoffel W. and Blau R. (1995) Rat and human glutamate transporter GLAST1 stable heterologous expression, biochemical and functional characterization. *Biol. Chem. Hoppe-Seyler* **376,** 511–514.

Storck T., Schulte S., Hofmann K., and Stoffel W. (1992) Structure, expression, and functional analysis of a Na^+-dependent glutamate/aspartate transporter from rat brain. *Proc. Natl. Acad. Sci. USA* **89,** 10955–10959.

Tong G. and Jahr C. E. (1994) Block of glutamate transporters potentiates postsynaptic excitation. *Neuron* **13,** 1195–1203.

Zerangue N., Arriza J. L., Amara S. G., and Kavanaugh M. P. (1995) Differential modulation of human glutamate transporter subtypes by arachidonic acid. *J. Biol. Chem.* **270,** 6433–6435.