PKC induces internalization and retention of the EAAC1 glutamate transporter in recycling endosomes of MDCK cells

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Padovano V, Massari S, Mazzucchelli S, Pietrini G. PKC induces internalization and retention of the EAAC1 glutamate transporter in recycling endosomes of MDCK cells. Am J Physiol Cell Physiol 297: C835–C844, 2009. First published July 15, 2009; doi:10.1152/ajpcell.00212.2009.—Here we show that stimulation of protein kinase C (PKC) by phorbol 12-myristate 13-acetate (PMA) treatment induces a time-dependent decrease in glutamate transport activity due to relocalization of the excitatory amino acid carrier 1 (EAAC1) glutamate transporter from the apical surface of polarized epithelial Madin-Darby canine kidney (MDCK) cells to intracellular compartments. The PKC-induced internalization of EAAC1 is negatively regulated by the calcineurin inhibitor cyclosporine A and by the expression of a dominant-negative mutant of the endocytic protein dynamin 1, a well-known target of the phosphatase activity of calcineurin. Using [32P]-metabolic labeling experiments, we found unchanged levels of phosphorylated EAAC1, indicating that EAAC1 relocalization does not depend on PKC and calcineurin modification of the transporter, while we found that a target of these modifications was the serine79 residue of dynamin, a calcineurin substrate that in its dephosphorylated form activates the endocytic functions of dynamin. These data suggest that PMA stimulates endogenous dynamin and that this activation is required to mediate internalization of EAAC1 in MDCK cells. By immunofluorescence experiments with endosomal markers we demonstrated that internalized EAAC1 accumulates in endosomes also containing the basolateral betaine-GABA transporter BGT1 and activated PKCo. The sustained activation of PKC was required to maintain the transporters in the endosomal compartment, while a posttreatment with a PKC-specific inhibitor induced the recycling of the transporters to their appropriate surfaces. Taken together, our data indicate that PKC activity regulates EAAC1 surface density in MDCK cells by inducing its internalization and retention in PKCo-labeled recycling endosomes common to apical and basolateral proteins.

common recycling endosomes; cyclosporine A; dynamin 1; pericentrion; protein kinase Co; excitatory amino acid carrier

GLUTAMATE IS THE PRIMARY EXCITATORY NEUROTRANSMITTER IN THE CENTRAL NERVOUS SYSTEM. Increased levels of extracellular glutamate induce excitotoxicity and may contribute to neuronal damage in neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, and multiple sclerosis (34).

A family of Na+-dependent glutamate transporters localized to the plasma membrane mediates reuptake of extracellular glutamate, thus ensuring appropriate excitatory signaling and limiting the excitotoxic potential of glutamate in the mammalian central nervous system. Among the glutamate (or excitatory amino acid) transporters, the GLT1 and the glutamate/aspartate transporter GLAST (human EAAT2 and EAAT1) are predominantly expressed in astroglial cells (27, 36), whereas excitatory amino acid carrier (EAAC) 1 (human EAAT3), EAAT4, and EAAT5 are expressed in neuronal cells (4, 17).

Astroglial GLT1 and GLAST play a prominent role in the regulation of extracellular levels of glutamate, whereas the function of EAAC1 in the central nervous system is less clear. It has been recently found that EAAC1 functions as a neuronal cysteine transporter and that its dysfunction leads to impaired glutathione homeostasis and neurodegeneration (3). EAAC1 is also expressed in several extraneuronal tissues (21). In renal tubule cells, EAAC1 is the main system involved in the reuptake of glutamate and aspartate from urine, and glutamate transporter EAAC1-deficient mice develop dicarboxylic aminoaciduria (21), a very rare human disorder (MIM 222730).

To fulfill their physiologic responses, the transporters must localize on plasma membrane-specific domains. It has become increasingly clear that the activity of these transporters can be regulated acutely by mechanisms associated with a redistribution of transporters to or from the plasma membrane without changing the total number of transporters (31). The intracellular trafficking of many neurotransmitter transporters, including EAAC1, has been largely documented to be controlled by protein kinase C (PKC) activity (28). PKC regulation is independent of the total number of transporters and occurs within minutes, thus indicating that the process does not involve de novo synthesis and modifications occurring at the level of the endoplasmic reticulum, but rather recycling from and/or to the plasma membrane. The mechanisms governing PKC-induced transporter trafficking, however, are still not clear. In addition, different results have been published on PKC regulation of EAAC1, depending on the cell type expressing the transporter. In C6 glioma and primary neuronal cultures, the transporter localizes in intracellular pools and PKC activation increases its surface delivery (11) and association with the glutamate transporter-associated protein 3–18 (GTRAP3–18, also called adducin), a protein that negatively controls EAAC1 transport (1, 39). In polarized renal epithelial Madin-Darby canine kidney (MDCK) cells, EAAC1 localizes to the apical cell surface and PKC activation induces a reduction of transport activity (8, 37), but the mechanism is still unidentified.

By functional, morphological and biochemical assays we have investigated the mechanisms of PKC regulation of EAAC1 in MDCK cells and found that phorbol 12-myristate 13-acetate (PMA) activation of PKC negatively regulates the transport activity by decreasing surface EAAC1 through an internalization process that also requires calcineurin activity. Target of PKC and calcineurin modifications is endogenous dynamin(s) rather than the transporter, suggesting that the...
internalization of the transporter depends on calcineurin-mediated activation of the endocytic activity of endogenous dynamin(s). Moreover, we found that PKC activity mediates the retention of the transporter in PKCα-labeled recycling compartments.

**MATERIALS AND METHODS**

**Cell cultures and transfections.** The cDNA encoding rabbit EAAC1 (38) was stably transfected in MDCK cell lines by calcium phosphate transfection and G418 selection as previously described (25). The MDCK strain II or the transfected MDCK-EAAC1 cell lines were seeded at a density of 4 × 10⁴ cells/cm² on glass coverslips for immunocytochemistry, or tissue culture dishes and multiwell plates (Falcon) for biochemistry and glutamate uptake experiments. Cells were cultured for 60 h to reach confluence before treatments. The MDCK-EAAC1 cell lines were transfected 48 h after being plated with cDNA coding for hemagglutinin (HA)-tagged K44A dynamin 1, a kind gift of Dr. S. L. Schmid (10); PKCα, a kind gift of Dr. G. P. Perletti (24); BGTΔS; or myc-BGT1 (23).

**Cell treatment.** The cells were starved in Dulbecco’s modified Eagle’s medium containing 25 mM HEPES for 30 min at 37°C and were then treated either with the indicated concentration of PMA (Sigma, Milan, Italy) for 30 min (unless otherwise indicated) to stimulate PKC or with 2 μM GF109203X (GF; Tocris, Bristol, UK) for 20 min before PMA treatment to prevent PKC activation or 60 min after PMA treatment to inhibit PKC activation. Dynamin 1 and 2 were inhibited by 60 min of pretreatment with 60 μM Dynasore (Sigma) before PMA treatment, and clathrin-mediated endocytosis was blocked by 30 min of pretreatment with 0.45 M hypertonic sucrose. To inhibit protein phosphatase 1 (PP1) and 2A (PP2A) or calcineurin [protein phosphatase 2B (PP2B)], the cells were pretreated with 1 μM okadaic acid (Sigma) or 100 μM cyclosporine A (CSA; Novartis Pharma, Basel, Switzerland) before PMA stimulation. As CSA was dissolved in 0.1% ethanol, the effect of ethanol was also tested. After treatments, the cells were processed for immunofluorescence or biochemical and functional studies, as indicated.

**Primary antibodies.** Affinity-purified rabbit polyclonal antibody was raised against synthetic peptide consisting of amino acids 500–523 of the COOH termini of rabbit EAAC1 (38). No cross-reactivity of the EAAC1 affinity-purified antibody with endogenous canine EAAC1 was observed in the MDCK cell lines. The following antibodies were also used: 9E10 monoclonal antibody directed against the c-myc epitope (MBL, Santa Cruz Biotechnology, Santa Cruz, CA) to recognize the myc-tagged BGTΔS/BGT1, monoclonal anti-Na⁺–K⁺-ATPase pump as a marker of the lateral domain of epithelial cells (25), polyclonal antibodies against the bovine mannose-6-phosphate receptor (MPR) as a late endosomal marker (a gift from Dr. B. Hoflack) (13) and human cathepsin D (CatD) as a lysosomal marker (a gift from Dr. C. Isidoro) (16), monoclonal anti-HA (Roche) to recognize the HA-tagged dynamin 1 K44A, polyclonal antibody against dynamin 1 phosphorylated at serine775 (Chemicon International), PKCα H-7 monoclonal antibody (Santa Cruz Biotechnology), monoclonal anti-actin clone AC-40 (Sigma), and polyclonal anti-dynamin 1 (Affinity BioReagents, Golden, CO).

**Immunocytochemistry.** The cells were fixed in ice-cold methanol and permeabilized with 0.5% Triton X-100. Immunostaining with primary antibodies was followed by incubation with rhodamine-conjugated anti-rabbit and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Jackson ImmunoResearch). In the double-staining shown in Fig. 4B, the cells were first stained using the anti-MPR or anti-CatD antibodies, followed by rhodamine-conjugated anti-rabbit IgG, and then stained using the EAAC1 antibody followed by FITC-conjugated anti-rabbit IgG. To avoid binding of the FITC-conjugated secondary antibodies to free MPR or CatD antibodies, the cells were incubated in an excess of anti-rabbit Fab (12 mg/ml) before the EAAC1 staining.

For concanavalin A (ConA) labeling of surface glycoproteins, the MDCK-EAAC1 cell line was incubated on ice for 60 min with biotin-ConA (Sigma) in phosphate-buffered saline (PBS) containing 0.1 mM Ca²⁺ and 1 mM Mg²⁺. To reveal the lectin-bound surface glycoproteins, cells were stained with Texas red-conjugated streptavidin. The confocal images were obtained using a Bio-Rad MRC-1024 confocal microscope.

**Phosphorylation and immunoprecipitation experiments.** Metabolic labeling with [³²P]Pi, was carried out by preincubating cells in phosphate-free medium for 3 h (30) followed by incubation for 30 min at 37°C with phosphate-free medium containing [³²P]Pi (final specific activity 1 mCi/ml). The cells treated as described above were washed twice with cold phosphate-buffered saline and solubilized in lysis buffer (25 mM Tris·HCl, pH 7.4, 100 mM NaCl, 1 mM MgCl₂, 1% Triton X-100, 0.02% SDS, 5 mM DTT, 0.1 mM PMSF, and a mixture of protease and phosphatase inhibitors). After centrifugation at 12,000 g for 20 min, the lysates were incubated overnight at 4°C with antibodies preincubated for 2 h at 4°C with 20 μl of protein A-Sepharose (Sigma). The immunocomplexes bound to the beads were recovered by centrifugation, washed three times with lysis buffer, and loaded onto an 11% SDS-polyacrylamide gel. For analysis of the phosphorylated EAAC1, gels were dried and autoradiographed, whereas for protein analysis, gels were blotted onto nitrocellulose and probed with specific antibodies. The signal density was quantified using National Institutes of Health Image 1.59 software, and statistical significance was determined using the Student’s t-test.

**Glutamate uptake and cell surface biotinylation.** Cells (4 × 10⁴) were seeded on 24-mutiwell plates, grown for 60 h, and treated as indicated. Glutamate influx was assayed according to Yamauchi et al. (41) with modifications (26). Briefly, [¹⁴C]glutamate (Amersham Biosciences, Upsala, Sweden) in incubation buffer (125 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES pH 7.5) was applied at a final concentration of 0.5 μM for 10 min at room temperature, unless otherwise indicated. Uptake was terminated by aspirating the medium, and the cells were washed three times with ice-cold incubation buffer. After cell solubilization in 0.2 ml of 1% SDS, the samples were counted in 5 ml of scintillation solution (Ultima Gold, Packard, CA) using a beta counter. Biotinylation of apical proteins was carried out with 1 mg/ml Sulfo-NHS-LC-Biotin (Pierce Chemical, Rockford, IL) according to Sargiacomo et al. (33). The biotinylated surface proteins were allowed to bind to streptavidin beads (Sigma) for >3 h and were then separated from the supernatant by centrifugation. After being washed, the biotinylated proteins were released from the beads by incubation with 2× SDS loading buffer, separated by 9% SDS-PAGE, and transferred onto nitrocellulose (Schleicher and Shull, Dassel, Germany).

**RESULTS**

**PKC and calcineurin influence EAAC1 glutamate transport in MDCK cells.** Epithelial MDCK cells were used as a cell model to investigate the kinase and phosphatase regulation of glutamate transporter activity. The EAAC1 glutamate transporter is endogenously expressed in MDCK cells, where it localizes to the apical surface (8). Since antibodies raised against rabbit EAAC1 used in this study do not recognize the canine transporter, this study has been performed in MDCK cell lines stably expressing the cDNA encoding rabbit EAAC1 (38).

To investigate whether PKC regulates the activity and localization of EAAC1, we carried out [³H]glutamate uptake and immunofluorescence experiments in MDCK-EAAC1. A similar reduction in glutamate influx after stimulation with the general PKC activator PMA was observed in untransfected MDCK and MDCK-EAAC1 cells (Fig. 1). Glutamate uptake in
MDCK-EAAC1 is predominantly due to the transfected transporter, as [3H]glutamate uptake is fivefold higher in MDCK-EAAC1 than in parental MDCK cells (Fig. 1A). In addition, we observed a dose-dependent effect on transport activity after incubation with PMA at a concentration higher than 1 nM, and an optimal effect was obtained with 20 nM PMA in both parental and transfected MDCK-EAAC1 cells (~30% reduction). The PMA-mediated effect was prevented by preincubation with the GF specific inhibitor of PKC (Fig. 1B and C), thus further indicating a PKC-mediated regulation of transport activity. These effects were not due to the 0.2% DMSO used to dissolve PMA and GF, as DMSO alone did not influence glutamate transport activity (data not shown).

To test whether phosphatases are involved in EAAC1 transporter modulation, parental MDCK and MDCK-EAAC1 cells were pretreated for 30 min with 1 μM okadaic acid to inhibit PP1 and PP2A or with 100 μM CsA to block PP2B (also called calcineurin) activity. Surprisingly, CsA, the specific inhibitor of calcineurin activity, prevented the PMA-induced decrease in transport activity rather than increasing the PMA effect (Fig. 1B and C). This was not due to the 0.1% ethanol used to dissolve CsA, because ethanol alone did not prevent the PMA-mediated reduction of EAAC1 transport. It is interesting to note that a 30-min treatment with CsA in the absence of PMA stimulation significantly increased EAAC1 activity in MDCK and MDCK-EAAC1 cells (Fig. 1C), suggesting that also the basal activity of EAAC1 is regulated by calcineurin. These analyses therefore revealed that endogenous and transfected EAAC1 have a similar behavior and indicate that the PKC-negative effect on the transporter’s function requires the activity of calcineurin.

PKC and calcineurin activities relocate apical EAAC1 to intracellular compartments. Reduction of transport activity has been previously observed by Trotti and colleagues (37), but it was unclear whether it was due to decreased ligand affinity or...
downregulation of cell surface transporters. To verify whether PMA-induced decrease in glutamate influx is associated with reduced surface localization, we analyzed the transporter distribution by confocal laser microscopy in MDCK-EAAC1 cells (Fig. 2). In horizontal sections, the EAAC1 antibody revealed a typical punctuate staining characteristic of proteins localized in the microvillar apical surface in polarized MDCK cells. This distribution was affected by PMA treatment, because the transporter accumulated in large subapical spots. In contrast, PMA treatment did not affect the distribution of the lateral marker Na\(^{+}\)-K\(^{+}\)-ATPase (green staining). Pretreatment of the cells with GF prevented PKC-mediated relocalization of EAAC1, thus maintaining the apical staining. In addition, CsA but not okadaic acid counteracted the PMA-induced relocalization of the apical EAAC1, suggesting an involvement of calcineurin in PKC-mediated regulation of EAAC1 activity and localization. The immunofluorescence data therefore consolidate the uptake data and together suggest that PMA-mediated decrease in glutamate transport activity is due to a decrease in EAAC1 cell surface distribution.

**PKC and calcineurin-dependent modifications of dynamin 1 are associated with internalization of EAAC1.** To assess whether PMA-induced relocation of EAAC1 is due to internalization of the transporter, we interfered with the main pathways of endocytosis by preventing clathrin coat formation with hypertonic sucrose (14), and dynamin-mediated fission of endocytic vesicles with Dynasore (19). The apical localization of EAAC1 was clearly maintained in cells treated with PMA and hypertonic sucrose or PMA and Dynasore (Fig. 3A). In these cells, intracellular EAAC1 localization was virtually never observed, indicating that PKC activation causes internalization of the transporter through dynamin and clathrin-dependent pathways.

To further demonstrate that PMA induces endocytosis of EAAC1 and to quantify the amount of transporter internalized, we performed a cell surface biotinylation assay. A decrease in biotinylated transporter was measured in cells treated with PMA, and the reduction was PKC dependent because the GF inhibitor prevented the PMA effect (Fig. 3B). The decrease in biotinylated transporter (~30%) entirely accounts for the reduction in glutamate uptake observed after PMA treatment (see Fig. 1), further demonstrating that PMA mediates the internalization of EAAC1 rather than altering the transporter ligand affinity.

To study whether the internalization of the transporter and its relocation to intracellular compartments were associated with modifications of the transporter mediated by PKC and/or calcineurin, MDCK-EAAC1 cells were metabolically labeled with \(^{32}\)P\(_{i}\) and then stimulated with PMA. As positive control, parallel experiments were performed in MDCK cells expressing the BGT1 epithelial GABA transporter (20). A low basal level of phosphorylated EAAC1 was revealed in control cells, which did not significantly change after PMA treatment at any of the experimental times and conditions tested (Fig. 3C).

These results suggested that PKC and calcineurin activities may exert their effects on EAAC1 relocalization through an indirect mechanism based on modifications of the endocytic machinery. We therefore analyzed whether PKC and calcineurin interfered with trafficking of bulk apical glycoproteins by an endocytosis morphological assay. In this assay, glycoproteins were labeled with biotinylated-ConA at 0°C, chased at
37°C for 30 min in the presence of 20 nM PMA, and then fixed and stained for EAAC1 (Fig. 3D). After a 30-min chase at 37°C without PMA, EAAC1 maintained its apical localization while ConA-labeled glycoproteins localized in intracellular structures. EAAC1 and labeled glycoproteins colocalized in intracellular structures during the chase with PMA, thus indicating that EAAC1 is in endosomal compartments containing bulk apical glycoproteins. Interestingly, the calcineurin inhibitor CsA also affected the internalization of lectin-bound apical glycoproteins, strongly suggesting that the target of calcineurin activity is the internalization machinery of apical proteins rather than the transporter.

To investigate whether calcineurin activity is also required in PKC-mediated regulation of transporters localized to the basolateral surfaces, we analyzed the effect of CsA in the PMA-induced internalization of the basolateral GABA transporter BGT1 (20), and of the nonpolarized glutamate transporter GLT1 (8, 38). A 30-min PMA treatment caused a relocation of these transporters from the lateral surface to intracellular spots morphologically indistinguishable from those containing EAAC1 (Fig. 3E, compare the EAAC1 staining in Fig. 2). However, pretreatment of the cells with CsA did not maintain the staining of the lateral surface, thus indicating that calcineurin is not involved in the PMA-mediated relocation of transporters localized to the basolateral surface.

EAAC1 shares 50–60% sequence similarity with GLT1 but contains an apical localization signal located in its COOH-terminal domain that is responsible for its polarized localization; the substitution of this region with the related region of GLT1 causes nonpolarized distribution of EAAC1, and, on the contrary, the EAAC1 tail is sufficient to redirect GLT1 to the apical surface (8, 34, 38). To further investigate whether only apical transporters require calcineurin activity, we analyzed the effect of PMA and CsA on chimeric EAAC1 and GLT1 transporters having their cytosolic tails reciprocally exchanged (Fig. 3E). The EAAC-GLT chimera, which lacks the predominant apical localization of EAAC1, relocates intracellularly in response to PMA stimulation, but this relocation was independent on calcineurin activity. On the contrary, the PMA-mediated relocation of the apically targeted GLT-EAAC chimera was dependent on calcineurin activity. Taken together, these data indicate that calcineurin activity is required only for PMA-stimulated internalization of transporters localized to the apical surface.

It is known that calcineurin is key to the activity-dependent stimulation of bulk endocytosis pathway in neuronal cells, through dephosphorylation of a set of endocytic proteins called dephosphins (9); within this group there is dynamin 1, a key regulator of synaptic vesicle fission (22). Among the three isoforms of dynamin expressed in mammals, dynamin 2 is the major isoform (10) in MDCK-EAAC1 cells. As expected, DNDyn1 showed a punctate staining typical of apical proteins, and transfected cells maintained the apical staining of EAAC1 after PMA treatment [Fig. 3F, compare magenta staining in cells expressing DNDyn1 (green) with that in untransfected cells]. To investigate whether dynamin 1 is expressed in MDCK cells, we stained a total cell extract with a dynamin 1-specific antibody and found that the antibody recognized a band of the expected molecular weight that remained unaltered regardless of the treatments. As it is known that phosphorylation on serine778 of dynamin 1 mediated by kinase Cdk5 decreases the endocytic activity of dynamin 1, while dephosphorylation of this residue mediated by calcineurin reactivates dynamin 1 function (35), we stained MDCK cell extracts with an antibody against phospho-serine778. The antibody revealed a markedly reduced band after 30 min of stimulation with PMA (60% of the control) that was completely abolished by the GF inhibitor, and, even more interesting, also the calcineurin inhibitor CsA prevented the dephosphorylation of endogenous dynamin (Fig. 3G). These results indicate that dynamin 1 and/or dynamin 2 in MDCK cells is regulated by PKC and calcineurin.

PKC and calcineurin activities relocate apical EAAC1 to endosomes containing proteins recycling to the basolateral surface. To investigate the nature and functions of the intracellular compartment containing internalized EAAC1, we first assessed the kinetics of EAAC1 relocation to these structures by [3H]glutamate uptake and immunofluorescence experiments performed in parallel. We measured a progressive reduction of [3H]glutamate transport starting from 5 min and reaching a maximal effect after 25–30 min of incubation with 20 nM PMA (Fig. 4A, left). The progressive reduction of transport activity paralleled the increased relocalization of EAAC1 to intracellular compartments (Fig. 4A, right). Horizontal confocal sections taken at the subapical perinuclear region clearly showed an EAAC1-positive staining after a 10-min treatment with 20 nM PMA, which increased in intensity up to 30 min. Treatments for longer periods of time or with increased concentration of PMA did not apparently change any further the EAAC1 distribution (data not shown). The progressive reduction of uptake might therefore be compensated by the accumulation of the transporter in endosomal compartments, further confirming the association between removal from the cell surface and intracellular accumulation of EAAC1.

To characterize the endosomal compartment containing EAAC1, we performed double-immunofluorescence staining with markers of endocytic and degradative endosomes. Staining with specific antibodies failed to reveal EAAC1 colocalization with the early endosome-associated protein 1 (EEA1), the MPR late endosomal marker, or the CatD lysosomal marker (Fig. 4B). These experiments excluded a localization of EAAC1 in early endosomes and along degradative pathways, and we therefore investigated whether EAAC1 is targeted to recycling compartments after PMA treatment. In MDCK cells, apical recycling proteins may accumulate in the compartment for the common recycling of apical and basolateral proteins (common recycling endosomes, CREs) or in the endosomal compartment for the exclusive recycling of apical proteins (apical recycling endosomes, AREs) (29). To clarify whether EAAC1 is targeted to CREs or AREs, we took advantage of previous results obtained with a truncated form of the BGT1 epithelial GABA transporter (BGTΔ5), a mutant transporter located in basolateral surfaces and recycling compartments (23). In control conditions, the myc-tagged BGTΔ5 transiently transfected in MDCK-EAAC1 cells accumulated in intracellular perinuclear spots devoid of EAAC1 staining (Fig. 4C,
PKC REGULATION OF EAAC1

A

B

C

D

E

F

G

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On the contrary, EAAC1 colocalized with the BGTΔ5 after PMA treatment. Thus, the intracellular compartment containing the apical EAAC1 and the basolateral recycling truncated transporter is bona fide identified as the CREs. To further demonstrate the recycling nature of the compartment, we investigated whether the transporters were able to recycle to respectively the apical and basolateral surface. To this end, we switched off the PKC signaling by posttreating the cells with GF. In these experiments, MDCK-EAAC1 cells were transiently transfected with the full-length BGT1, which contrarily to the BGTΔ5 mutant relocates from the lateral surface to the recycling compartment only after PMA stimulation (20). Figure 4D shows that EAAC1 and BGT1 colocalize after PMA treatment and that the inhibition of PKC induces the recycling of the transporters to their appropriate opposite plasma membrane, further indicating the common recycling nature of the intracellular compartment containing EAAC1 and BGT1 and that sustained PKC activity is required to accumulate the transporters in this compartment.

Sustained activation of PKC retains EAAC1 in PKC-positive recycling endosomes. It has been shown that on persistent stimulation with 100 nM PMA (30–60 min), classical PKC (α and βII) translocates in a juxtanuclear region named pericentrum (5), where recycling proteins become sequestered (15). To further investigate the nature of the compartment accumulating EAAC1, we performed double-immunofluorescence staining with EAAC1 and PKCα antibodies in MDCK-EAAC1 cells transiently expressing PKCo cDNA. Translocation of PKCo to intracellular compartments positively stained for EAAC1 was already observed after 30 min of treatment with 20 nM PMA (data not shown), but colocalization was unambiguously seen using 100 nM PMA (Fig. 4E). Translocation of PKCo from the cell surface to recycling endosomes (pericentrum) has been shown to depend on clathrin-dependent endocytosis and to be reversed by inhibitors of PKC (15), and we therefore inhibited clathrin-dependent pathways of endocytosis with hypertonic sucrose and reverted PKC activation by posttreatment with GF. A clear surface localization of EAAC1 and PKCo was observed with both treatments, thus suggesting a direct link between PKCo and EAAC1 distribution.

**DISCUSSION**

PKC activity has been documented to induce rapid changes in transport activity of the EAAC1 glutamate transporter, but conflicting results have been obtained depending on the cell type. For instance, in C6 glioma or primary neuronal cultures the transporter localizes to intracellular pools and PKC activation increases the delivery of the transporter to the cell surface (11). By contrast, in MDCK cells, EAAC1 has been shown to localize to apical surfaces and its transport activity to be decreased by PKC activation (8, 37). Consistent with these data, we here show that the reduced EAAC1 activity is not due to a decrease in ligand affinity but to a decrease in surface density due to its internalization and retention in recycling endosomes common to apical and basolateral transporters.

We found that PMA treatment progressively reduced glutamate transport to ~30% within 30 min. This reduction was entirely due to internalization of the transporter from the cell surface, as documented by a ~30% reduction in biotinylated surface transporter. The apical localization of EAAC1 was maintained in MDCK cells treated with the PKC specific inhibitor GF or with reagents that inhibit endocytosis, such as hypertonic sucrose and the chemical inhibitor Dynasore, or by expression of DNdyn1. Thus, PKC activation triggers downregulation of EAAC1 activity by decreasing the apical surface density of the transporter.

Our data indicate that PKC-mediated internalization of EAAC1 is also dependent on calcineurin, a serine/threonine phosphatase implicated in brain development, synaptic plasticity, and neurodegenerative disorders (40). Calcineurin controls...
activity-dependent endocytosis in central nerve terminals through dephosphorylation of the dephosphin dynamin 1, a GTPase protein that mediates membrane fission to cause both clathrin-dependent and bulk endocytosis in neuronal cells (9). Dynamin 1 cycles between an active dephosphorylated state mediated by calcineurin and an inactive phosphorylated state mediated by the kinase cdk5 on serine774,778 (35). This cycle is regulated by calcium influx during synaptic activity that stimulates calcineurin activity, while cdk5 is constitutively active in axon terminals. We found that a target of modifications induced by PKC and calcineurin in MDCK cells was an endogenous dynamin. As already discussed, it is unknown whether MDCK cells together with the ubiquitous dynamin 2 also express the neuronal dynamin 1 isoform. However, the dynamin 1-specific antibody recognized a band of corresponding molecular weight, and the antibody against phosphoserine778 revealed that an endogenous dynamin is regulated by PKC and calcineurin. The identity of the dynamin isoform regulated by PKC and calcineurin at the apical surface of MDCK cells remains to be confirmed; however, these results may entirely or at least partially explain the EAAC1 internalization.

In support of a role for calcineurin in EAAC1 apical endocytosis, we found that relocalization of transporters located to the lateral surface was CsA insensitive, but they gained sensitivity to CsA when forced to localize to the apical surface. Although the mechanism of PMA stimulation of dynamin endocytic activity in MDCK cells is currently unknown, PKC activation might induce a local influx of calcium to activate calcineurin and/or might negatively control cdk5 kinase with a mechanism similar to that recently described by Sahin and colleagues (32). However, these potential mechanisms may account for the increased internalization of the apical EAAC1 but they appear dispensable in the internalization of basolateral transporters.
Another possible explanation of EAAC1 internalization is that PKC activity may also increase the affinity for EAAC1 binding to syntaxin 1A, a protein that has been shown to stimulate constitutive and kainic acid regulated clathrin-mediated internalization of EAAC1 in C6 glioma cells (42). However, syntaxin 1A is not expressed in MDCK cells, and in C6 cells it seems to be required to sort internalization of EAAC1 to early endosomes and lysosomes rather than to recycling compartments. MDCK cells express the isoform syntaxin 3A on the apical surface, and further experiments are needed to investigate the role of syntaxin 3A in EAAC1 internalization.

The PMA-induced trafficking of many neurotransmitter transporters is accompanied by significant global increases in transporter phosphorylation states (28). Mutagenesis of canonical PKC phosphorylation sites, however, failed to affect these relocalizations (7), suggesting that PKC-induced phosphorylation is not required for PKC-induced transporter relocalization but rather is an epiphenomenon due to the protein sequestration in a subcellular compartment in which the transporters might be phosphorylated. Our data indicate that EAAC1 phosphorylation does not occur either at the level of the plasma membrane or in the intracellular compartment, even though after PKC stimulation the transporter clearly colocalizes with the PKCα both at the plasma membrane and in the intracellular compartment.

Here we have shown that PKC activation also mediates the accumulation of EAAC1 in intracellular structures containing proteins recycling to the basolateral surface (the unstimulated mutant BGTΔ5 or the PKC-stimulated full-length BGT1) (20, 23). As EAAC1 and BGT1 localize to opposite plasma membrane domains, their intracellular colocalization identifies these compartments as CREs. On the other hand, we have shown that EAAC1 also colocalizes with PKCα in endosomes showing features similar to the pericentron identified by Becker and Hannun in human embryonic kidney 293 cells (5), and thus suggesting that pericentron may correspond to a subset of CREs in polarized MDCK cells. Evidence indicates that translocation of PKCs (α or βII) to pericentron is associated with sequestration of membrane-recycling components, a process dependent on persistent but reversible PKC activity to cause a clathrin-mediated translocation of PKCα to endosomal structures (5, 15). In MDCK cells we have shown that PMA stimulates clathrin-mediated translocation of PKCα to EAAC1-positive structures and that sustained activation of PKC is required to maintain the localization of PKCα and transporters (apical and basolateral) in these structures. Thus, our data support a role for activated PKCα in the retention of apical and basolateral proteins in a subset of CREs functionally related to the pericentron. Interestingly, a PKC-dependent association of EAAC1 and PKCα has been described in C6 glioma cells and rat brain synaptosomes (12), and the authors proposed a function of PKCα in triggering the distribution of EAAC1. Our data are consistent with this possibility but exclude that the association may depend on EAAC1 phosphorylation.

PKC-mediated relocalization of EAAC1 might also require modifications of protein–protein interactions retaining the transporter at the cell surface or in the recycling compartment. The GTRAP3–18/addiscin partner of EAAC1 at the plasma membrane of neuronal and nonneuronal cells (18) exhibits putative PKC phosphorylation motifs (6). However, PKC activity has been shown to increase rather than decrease GTRAP3–18 association to EAAC1 at the plasma membrane, and the GTRAP3–18/EAAC1 association has been described to negatively regulate EAAC1 activity without affecting the surface density of the transporter in C6 glioma cells and neurons (1, 39). Putative PKC phosphorylation motifs are also found in the ADP-ribosylation factor-like 6 interacting protein 1 (Arl6ip1), a partner of GTRAP3–18 in intracellular compartments that indirectly promotes a positive control of EAAC1 glutamate transport in C6 glioma cells (1). It is currently unknown whether GTRAP3–18–Arl6ip1 association is affected by PKC-mediated phosphorylation on GTRAP3–18 and/or Arl6ip1, and whether and how this regulation affects EAAC1 transport in MDCK cells.

Taken together, our data indicate that PKC stimulation negatively regulates EAAC1 activity in polarized MDCK cells by increasing the internalization of the transporter and its retention in recycling compartments containing apical and basolateral transporters. PKC-mediated internalization and retention of EAAC1 do not occur through a direct mechanism involving phosphorylation/dephosphorylation of the transporter and are associated with modifications of the level of phosphorylation of endogenous dynamin(s) and on translocation of PKCα.

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REFERENCES

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