Caveolin-1 Regulates the Delivery and Endocytosis of the Glutamate Transporter, Excitatory Amino Acid Carrier 1*

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The sodium-dependent glutamate transporter, excitatory amino acid carrier 1 (EAAC1), has been implicated in the regulation of excitatory signaling and prevention of cell death in the nervous system. There is evidence that EAAC1 constitutively cycles on and off the plasma membrane and that under steady state conditions up to 80% of the transporter is intracellular. As is observed with other neurotransmitter transporters, the activity of EAAC1 is regulated by a variety of molecules, and some of these effects are associated with redistribution of EAAC1 on and off the plasma membrane. In the present study we tested the hypothesis that a structural component of lipid rafts, caveolin-1 (Cav-1), may participate in EAAC1 trafficking. Using C6 glioma cells as a model system, co-expression of Cav-1 S80E (a dominant-negative variant) or small interfering RNA-mediated knock-down of caveolin-1 reduced cell surface expression of myc epitope-tagged EAAC1 or endogenous EAAC1, respectively. Cav-1 S80E slowed the constitutive delivery and endocytosis of myc-EAAC1. In primary cultures derived from caveolin-1 knock-out mice, a similar reduction in delivery and internalization of endogenous EAAC1 was observed. We also found that caveolin-1, caveolin-2, or Cav-1 S80E formed immunoprecipitable complexes with EAAC1 in C6 glioma and/or transfected HEK cells. Together, these data provide strong evidence that caveolin-1 contributes to the trafficking of EAAC1 on and off the plasma membrane and that these effects are associated with formation of EAAC1-caveolin complexes.

There are several observations that suggest an important role for EAAC1 in brain physiology. First, although initial studies showed no abnormalities in EAAC1-deficient mice, more recent studies have demonstrated that these animals develop age-dependent neurodegeneration attributed to depletion of glutathione and consequent oxidative injury (1, 2). Second, antisense-mediated knock-down of EAAC1 results in mild excitotoxicity and a seizure-like phenotype that apparently arises from decreased synthesis of the inhibitory neurotransmitter γ-aminobutyric acid (3, 4). Third, there is evidence that this post-synaptic transporter limits the spillover of glutamate to neighboring synapses (5). Fourth, preliminary studies suggest that knock-down of EAAC1 in the hippocampus followed by high frequency stimulation evokes long-term depression instead of long-term potentiation, and may impair learning in the Morris water maze (6).

Several studies have provided evidence that the activity and cell surface expression of EAAC1 can be rapidly regulated (for reviews, see Refs. 7 and 8). Activation of protein kinase C (PKC), platelet-derived growth factor (PDGF) receptor, or neurotensin receptor increases EAAC1 cell surface expression in primary cultured neurons and/or C6 glioma cells (9–13). In these same model systems, EAAC1 constitutively cycles on and off the membrane with a half-life of ~5–7 min (14). In vivo, a substantial amount of EAAC1 immunoreactivity is observed in intracellular compartments (15–17), consistent with the possibility that an intracellular pool of EAAC1 is available for redistribution to the plasma membrane in the intact nervous system. In fact, long-term potentiation or contextual fear conditioning increase the amount of EAAC1 observed in a subcellular fraction enriched in plasma membranes (18, 19). These studies provide strong evidence that EAAC1 trafficking is regulated and support the notion that EAAC1 trafficking may be important for synaptic plasticity.

Morphologically, caveolae are 50–100-nm plasma membrane invaginations observed in different cell types including adipocytes, endothelial cells, muscle cells, and fibroblasts. The members of the caveolin gene family are caveolin-1, -2, and -3. Interestingly, neurons express all three caveolins but lack morphologically defined caveolae (for review, see Ref. 20). Traditionally caveolae have been associated with endocytosis, but more recently caveolin has also been implicated in a variety of cellular functions, including targeting of proteins, assembly/or organization of signaling complexes, and lipid transport (for reviews, see Refs. 21–24).

Plasma membrane transporters appear to be regulated by caveolae/lipid rafts. The glutamate transporter EAAT2 is highly enriched in lipid rafts, and EAAT2 cell surface expression and activity are decreased by the disruption of these microdomains (25). Ciliary neurotrophic factor redistributes GLAST and GLT-1 to raft domains of activated astrocytes, and improves

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3 The abbreviations used are: EAAC1, excitatory amino acid carrier 1; ANOVA, analysis of variance; Cav-1, caveolin-1; Cav-2, caveolin-2; EAAT1–4, excitatory amino acid transporter subtypes 1–4; GTRAP, glutamate transporter interacting protein; Mes, 2-mercaptoethanesulfonic acid; PDGF, platelet-derived growth factor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SNAREs, soluble N-ethylmaleimide-sensitive attachment protein receptor; siRNA, small interfering RNA; PBS, phosphate-buffered saline; WT, wild type.
Regulation of EAAC1 Trafficking by Caveolin

glutamate handling in vivo (26). Pharmacological disruption of lipid rafts inhibits the PKC-dependent redistribution of serotonin transporters from the plasma membrane to an intracellular compartment (27). In preliminary studies, we found that depletion or disruption of cholesterol increased EAAC1 cell surface expression and slowed endocytosis, implicating caveole/lipid rafts in EAAC1 internalization (28). Because some studies have provided evidence that caveolin may play a key role in endocytosis of other membrane proteins (for reviews, see Refs. 29–32), we decided to pursue the hypothesis that caveolin might participate in trafficking of EAAC1.

In the present study, the effects of dominant-negative variants, siRNA-mediated knock-down, and knock-out of caveolin-1 on steady state cell surface expression, delivery, and internalization of EAAC1 were examined. Based on these analyses, we conclude that caveolin facilitates both the constitutive delivery and internalization of EAAC1. We also provide evidence that caveolin interacts with EAAC1, suggesting that this interaction may participate in the regulation of EAAC1 trafficking by caveolin.

EXPERIMENTAL PROCEDURES

Materials—Sulfosuccinimydyl-2-(biotinamido) ethyl-1,3-di-thiopropionate (sulfo-NHS-SS-biotin), sulfo-N-hydroxysuccinimidobiotin (sulfo-NHS-biotin), Ultralink® immobilized monomeric avidin beads, and the bicinchoninic acid (BCA) protein assay reagent kit were purchased from Pierce. The affinity purified rabbit polyclonal anti-EAAC1 antibody and the corresponding antigenic peptide were obtained from Alpha Diagnostics International (ADI, San Antonio, TX). A second anti-EAAC1 antibody was kindly provided by Dr. Jeffrey Rothstein (Johns Hopkins University). The anti-caveolin-1 and anti-caveolin-2 mouse monoclonal antibodies were purchased from Transduction Laboratories (San Diego, CA). The anti-transferrin receptor antibody was from Zymed Laboratories Inc. The rabbit anti-caveolin-1 polyclonal antibody was purchased from Santa Cruz (Santa Cruz, CA). The protein G-agarose beads were obtained from Invitrogen. Phorbol 12-myristate 13-acetate (PMA) and Mes-sodium salt (MesNa) were purchased from Sigma. The protein G-agarose beads were obtained from Invitrogen. Phorbol 12-myristate 13-acetate (PMA) and Mes-sodium salt (MesNa) were purchased from Sigma. The protein G-agarose beads were obtained from Invitrogen. Phorbol 12-myristate 13-acetate (PMA) and Mes-sodium salt (MesNa) were purchased from Sigma.

Cell Cultures—C6 glioma cells were obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin as previously described (9). Cells were incubated at 37 °C in 5% CO₂. This cell line endogenously expresses EAAC1 and none of the other subtypes of glutamate transporters (9). HEK-293T were obtained from the American Type Culture Collection and cultured similarly. All cell culture reagents were obtained from Invitrogen, except the defined fetal bovine serum that was purchased from HyClone (Logan, UT). C6 glioma were transfected using genePORTER reagent (GTS, San Diego, CA) as previously described (34). HEK-293T cells were transfected using the CalPhos transfection kit (Clontech) according to manufacturer’s specifications.

Neuron/Astrocyte-mixed Cultures—Caveolin-1 knock-out mice and genetic F1 control mice were obtained from Jackson Laboratories (strain: Cav1tm1Mls/J, stock number 004585) and bred in the animal facility of the Children’s Hospital of Philadelphia. Embryos (day 17–19) were removed from pregnant control and knock-out mice bred in parallel. After dissection and removal of the meninges and blood vessels, cortical tissue was incubated with trypsin for 25 min at 37 °C with intermittent agitation. After gentle trituration, 4 ml of cell suspension (400,000 cells/ml) were plated in Dulbecco’s modified Eagle’s medium/F-12 media supplemented with 1X B27, 10% fetal bovine serum, 6 g/liter glucose, 50 μg/ml streptomycin, and 50 units/ml penicillin onto a 6-cm dish that had been precoated with poly-D-lysine. Cultures were incubated in a 5% CO₂ incubator at 37 °C. After 2 days, the media was exchanged with Neurobasal media containing 1X B27, 0.5 mM l-glutamine, 5% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. Cultures were fed with a one-fifth medium exchange every 3–4 days and used after 12 days in vitro.

Measurement of EAAC1 Cell Surface Expression by Biotinylation—EAAC1 cell surface expression was measured as previously described (9, 35) with slight modifications. C6 gli-

3.1 (Invitrogen). The point mutation of Ser80 to Glu80 was prepared using QuikChange (Stratagene) following the manufacturer’s instructions. Caveolin-1 Δ80, a NH₂-terminal truncation of caveolin-1, was obtained by PCR using a 5’-primer that eliminates the 80 amino-terminal amino acids of caveolin-1. This primer also inserted the coding sequence for methionine at position 1. The sequences of all constructs were verified by sequencing.

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oma cells (grown in 10-cm dishes) or primary cultures (grown in 6-cm dishes) were first rinsed twice with ice-cold PBS containing 0.1 mM Ca\(^{2+}\) and 1.0 mM Mg\(^{2+}\) (PBS Ca\(^{2+}\)/Mg\(^{2+}\)), then cells were incubated with 2 ml (C6 cells) or 1 ml (primary cultures) of biotin solution (sulfo-NH-S-biotin at 1 mg/ml in PBS Ca\(^{2+}\)/Mg\(^{2+}\)) for 30 min at 4 °C with gentle agitation. After two rinses with PBS Ca\(^{2+}\)/Mg\(^{2+}\) containing 100 mM glycine, plates were incubated for 20 min at 4 °C to quench unreacted biotin. Finally, cells were lysed in RIPA buffer (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate containing protease inhibitors, including 1 mg/ml leupeptin, 250 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, and 1 mM iodoacetamide) for 1 h at 4 °C. After scraping, lysates were cleared of cell debris by centrifugation at 17,000 \(\times g\) for 20 min. An aliquot of cell lysate was saved and mixed with an equal volume of 4\(\times\) Laemmli buffer (240 mM Tris-HCl, pH 6.8, 8% SDS, 4% 2-mercaptoethanol) for further analysis as the “lysate fraction.” A second aliquot of cell lysate was incubated with an equal volume of UltraLink avidin-conjugated beads. After overnight incubation at 4 °C, supernatant containing non-biotinylated proteins (“intracellular fraction”) was recovered by centrifugation at 17,000 \(\times g\) for 15 min and mixed with an equivalent volume of 4\(\times\) Laemmli buffer. Beads containing the biotinylated (“cell surface proteins”) fraction were washed once with RIPA buffer, twice with a high salt buffer (50 mM Tris, 5 mM EDTA, 1% Triton X-100, pH 7.5), and once with a no salt buffer (50 mM Tris, pH 7.5). Finally, beads were incubated with 2\(\times\) Laemmli buffer (120 mM Tris-HCl, pH 6.8, 4% SDS, 2% 2-mercaptoethanol) for 30 min at 37 °C and centrifuged at 17,000 \(\times g\) for 15 min. The supernatant containing biotinylated proteins was saved for further analysis. The dilutions of all three fractions were the same so that the sum of the immunoreactivity found in the biotinylated and non-biotinylated fractions would equal that observed in the lysate fraction if the yield from extraction is 100%. Fractions were stored at −20 °C. The amount of protein in the lysate was determined using a BCA protein assay kit.

Measurement of EAAC1 Delivery to the Plasma Membrane—Cells were incubated in PBS containing biotin (4 ml for C6 cells and 2 ml for primary cultures at 1 mg/ml) at 37 °C as previously described (14, 36, 37). It is generally assumed that these conditions are permissive for normal intracellular trafficking (38). After different periods of time, cells were immediately cooled to 4 °C by washing the monolayer with ice-cold PBS Ca\(^{2+}\)/Mg\(^{2+}\) containing 100 mM glycine to both halt protein trafficking and quench unreacted biotin. In parallel, a control plate was incubated with ice-cold biotin (4 ml for C6 cells and 2 ml for primary cultures) for 30 min to quantify the amount of biotinylated transporter at the plasma membrane at time \(t = 0\) min. Extraction of biotinylated transporters was performed as described above. Previous studies have shown that the changes in biotinylated material do not result from the biotinylination of intracellular proteins due to the entrance of biotinylating reagent via endocytosis or pinocytosis (14, 36, 37, 39). The acylation reaction between the biotinylating reagent and primary amines of cell surface proteins is thought to be quite fast relative to the rate of typical endocytic events (Ref. 40 and 41, for discussion see Refs. 14 and 39). However, it is theoretically possible that some fraction of transporters could transit through the plasma membrane without being biotinylated, therefore the rate of delivery measured with this technique may represent an underestimate of the true rate.

Measurement of EAAC1 Endocytosis Using Reversible Biotinylation—Reversible biotinylation was performed as previously described (14, 36, 37). Cell surface proteins were biotinylated with the disulfide-containing reagent (sulfo-NHS-SS-biotin) and quenched as described above. Cells were rapidly rinsed with ice-cold PBS and ice-cold culture media. Except where indicated, they were then rinsed once with warmed (37 °C) culture media and then incubated in cell culture media for varying periods of time. Under these conditions, proteins undergo endocytosis. To halt internalization, cells were rinsed twice with ice-cold sodium-Tris (NT) buffer (150 mM NaCl, 1 mM EDTA, 0.2% bovine serum albumin, 20 mM Tris, pH 8.6). Biotin was “stripped” from cell surface proteins by incubating the cells twice for 25 min at 4 °C with the membrane impermeant disulfide reducing reagent, MesNa (50 mM, freshly dissolved in NT buffer). Two controls were used in each experiment. A plate of cells was incubated with NT buffer without MesNa to measure total surface expression of EAAC1. A second plate was used to control for the efficiency of stripping. These cells were not re-warmed prior to incubation with MesNa, and this sample was labeled as “0 min.” All stripping occurred at the same time. Biotinylated proteins were batch extracted as described above.

We used a variety of reagents and different concentrations of these reagents, including glutathione, Bond-Breaker™ TCEP solution, or MesNa. The maximal stripping observed (without re-warming) was 70–80%. The amount of transporter internalized at various times was calculated by subtracting the amount of biotinylated transporter observed at time \(t = 0\) after stripping (no re-warming) from the amount of biotinylated transporter observed after various periods of re-warming. This was expressed as a percentage of the amount of un-stripped transporter subtracted from the total amount of biotinylated transporter. These data were fit to a first-order decay process. Because the un-stripped pool of EAAC1 is also likely internalized, the rate calculated under these conditions is an underestimate of the rate of endocytosis. With the comparisons conducted (Cav-1 WT versus Cav-1 S80E or control versus caveolin-1 knock-out cultures), the percentage of strip was not significantly different between groups (see figure legends). Therefore, the differences in rates of endocytosis observed in the present study are independent of the artifact introduced by incomplete strip.

Immunoprecipitation—C6 or HEK-293T cells were washed with ice-cold PBS and lysed in 1 ml of the RIPA buffer (see above) modified to contain only 0.5% sodium deoxycholate and no SDS. Plates were agitated on a rotating orbiter for 1 h at 4 °C. Cell lysates were centrifuged at 17,000 \(\times g\) to remove cell debris. Lysates were pre-cleared with 40 \(\mu\)l of protein G-agarose beads and gentle shaking for 1 h at 4 °C. Approximately 1 mg of cell lysate protein was incubated overnight at 4 °C with 3 \(\mu\)g of affinity purified rabbit polyclonal anti-EAAC1 antibody (ADI). Immune complexes were recovered by incubation with 25 \(\mu\)l of protein G-agarose beads for 2 h at 4 °C and washed four times.
Regulation of EAAC1 Trafficking by Caveolin

Effects of Dominant-negative Variants of Caveolin on Surface-expressed EAAC1—One of the major structural components of caveolae is the protein caveolin (for reviews, see Refs. 29–32). There are three variants of caveolin, but only caveolin-1 and -2 are observed in C6 glioma (43). Two mutant variants of caveolin-1 have been developed to interfere with its function. The first, Cav-1 S80E, mimics phosphorylation of caveolin (44), and increases cell surface expression of the glucose transporter 4 presumably by inhibiting endocytosis (33). The second, Cav-1 Δ80, disturbs the formation of caveolin-1-containing lipid rafts (45). For our first series of experiments, the myc epitope-tagged human Cav-1 S80E was co-expressed with a myc epitope-tagged EAAC1. The values for biotinylated myc-EAAC1 (30 ± 3%) observed in cells co-transfected with empty vector are in agreement with the values obtained in previous reports for myc-EAAC1 (24–35%) and wild-type EAAC1 (20–30%) (9, 11, 39, 46). Therefore, we predicted that if Cav-1

**RESULTS**

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**FIGURE 1.** Co-expression of a dominant-negative mutant of caveolin reduces myc-EAAC1 cell surface expression. A, the top panel is a representative Western blot of total (cell lysate) and cell surface (biotinylated) myc-EAAC1 in C6 cells co-transfected with pcDNA, Cav-1 WT, Cav-1 S80E, or Cav-1 Δ80 cDNAs. In the lower panel, total myc-EAAC1 and cell surface myc-EAAC1 were calculated as a percentage of that observed in cells co-transfected with pcDNA (mean ± S.E., n = 8). In cells co-transfected with pcDNA the amount of biotinylated actin was 7 ± 4% (mean ± S.E.), and biotinylated actin did not change after co-transfection of the dominant-negative constructs. B, data in A were normalized to the amount of immunoreactivity observed in the lysate and expressed as a percentage of the immunoreactivity observed in cells co-transfected with pcDNA. Asterisk indicates a p < 0.05 compared with cells co-transfected with pcDNA or Cav-1 WT by ANOVA using a Bonferroni post hoc analysis.
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S80E inhibited endocytosis of EAAC1, it would increase the percentage of the transporter on the cell surface under steady state conditions. Compared with cells co-transfected with myc-EAAC1 and pcDNA, Cav-1 WT did not change the plasma membrane expression of myc-EAAC1 (95 ± 12%, mean ± S.E. of six independent experiments, data not shown). However, co-expression of Cav-1 S80E reduced myc-EAAC1 plasma membrane expression to 68 ± 8% of pcDNA-transfected cells (mean ± S.E. of six independent experiments, p < 0.05, by ANOVA with a post hoc test), but had no effect on total myc-EAAC1 expression (102 ± 6% for Cav-1 WT and 96 ± 12% for Cav-1 S80E). Given that some have suggested that caveolin may be important for endocytosis (for reviews, see Refs. 22, 30, 32, and 47), we were concerned that the myc-epitope might interfere with the dominant-negative effect or that expression of the human homolog in a rat cell line might influence the results.

To address these concerns, un-tagged versions of rat cDNAs for Cav-1 WT, Cav-1 S80E, and Cav-1 Δ80 were co-expressed with myc-EAAC1. Compared with control cells co-transfected with empty vector (pcDNA), co-transfection with Cav-1 S80E significantly reduced the amount of myc-EAAC1 found in the biotinylated (cell surface) fraction to ~60% of control (Fig. 1A). Because there was a modest but significant change in total myc-EAAC1 expression, the amount of biotinylated transporter was normalized to total myc-EAAC1 expression and expressed as a percentage of control (mock transfected cells). The percentage of total transporter found at the cell surface was also reduced by co-expression with Cav-1 S80E (Fig. 1B). The Cav-1 Δ80 construct had no significant effect on cell surface expression of myc-EAAC1; this was not pursued further. Together, these data provide strong evidence that both the human and rat homologues of the Cav-1 S80E variant of caveolin-1 reduce cell surface expression of myc-EAAC1.

Effects of Dominant-negative Caveolin-1 on the Kinetics of Delivery of EAAC1—A reduction in the steady state levels of EAAC1 at the plasma membrane implies that either the rate of constitutive delivery of EAAC1 to the plasma membrane is reduced or the rate of endocytosis is increased. To address these possibilities, myc-EAAC1 was co-expressed with either WT Cav-1 or Cav-1 S80E, and the rate of delivery of myc-EAAC1 to the plasma membrane was measured by incubating transfected C6 glioma with biotinylating reagent at 37 °C (trafficking permissive conditions) (14, 37). In cells co-transfected with myc-EAAC1 and Cav-1 WT, the amount of biotinylated myc-EAAC1 increased to 239 ± 30% of that observed at time 0 by 15 min. These observations are similar to those previously reported for the delivery of endogenous EAAC1 in C6 cells and consistent with a half-life for transporter at the plasma membrane of 5-7 min (14).

For the initial analyses, we assumed that the rate of increase in biotinylated myc-EAAC1 was dependent upon the size of the non-biotinylated (intracellular pool). Under these conditions, the rate of increase in biotinylated transporter was linear to at least 30 min, but by 60 min starts to plateau (Fig. 2, A and B). The rate of increase in biotinylated myc-EAAC1 was significantly slower in cells transfected with Cav-1 S80E compared with cells co-transfected with Cav-1 WT (Fig. 2C).

It is possible that after the transient transfection of the C6 cells the exocytic pathway may become saturated. If this were the case, the rate of appearance of biotinylated transporter would be independent of the size of the intracellular pool (following zero-order kinetics). Therefore, these same data were also analyzed without normalizing to the amount of non-biotinylated transporter. Using this approach, the rate of myc-EAAC1 delivery was also reduced in cells co-transfected with the Cav-1 S80E mutant to 53 ± 9% of that observed in cells transfected with Cav-1 WT (data not shown, p < 0.01, Student’s t test).

Effects of Dominant-negative Caveolin-1 on the Kinetics of EAAC1 Endocytosis—To determine whether Cav-1 S80E changes transporter endocytosis, myc-EAAC1 internalization was examined using a reversible biotinylation assay (Fig. 3A). The amount of myc-EAAC1 inaccessible to the membrane impermeant disulfide-reducing reagent increased with time (Fig. 3A). The net increase in inaccessible biotinylated myc-EAAC1 was expressed as a percentage of the total pool of transporter that could be stripped, and these data were fit to a first-order decay process (Fig. 3B). The half-life for endocytosis of cell surface myc-EAAC1 was significantly longer in cells co-transfected with Cav-1 S80E compared with cells co-transfected with Cav-1 WT (Fig. 3C). The half-life observed in cells expressing Cav-1 WT (~14 min) was somewhat slower than that predicted from the measure of delivery (see above). This longer half-life could either be due to rapid recycling of transporters back to the plasma membrane or to the fact that we were unable to strip ≥90% of cell surface transporters without...
Regulation of EAAC1 Trafficking by Caveolin

Caveolin-1 S80E slows endocytosis of myc-EAAC1. Internalization of myc-EAAC1 was examined in C6 cells using a disulfide-containing biotinylating reagent as described under "Experimental Procedures." A, representative Western blots showing the amount of biotinylated myc-EAAC1 immunoreactivity under the different conditions, the lower panel is a representative Western blot of the immunoreactivity in the total cell lysate. A plate was used to measure the pool of myc-EAAC1 available for internalization (no stripping) (T) and a second plate was not re-warmed prior to stripping (0 min). 5, 10, and 15 represent the times of rewarming. In these experiments, the percentage un-stripped at time 0 min was 31 ± 4% in cells co-transfected with Cav-1 WT and 28 ± 4% in cells co-transfected with Cav-1 S80E. B, the net increase in inaccessible biotinylated myc-EAAC1 was expressed as a percentage of the total pool of transporter that could be stripped, and these data were fit to a first-order decay process. C, half-lives of internalization of myc-EAAC1. These values were calculated from the rates obtained in five independent experiments (mean ± S.E.). Asterisk indicates a p < 0.01 compared with Cav-1 WT by Student's t test.

siRNA knock-down of caveolin-1 reduces EAAC1 cell surface expression. A, representative blot and summary of the effects of Cav-1 43 or Cav-1 37 siRNA on caveolin-1 expression. Double asterisk indicates p < 0.01 and triple asterisk indicates p < 0.001 compared with cells transfected with a control siRNA by ANOVA using a Bonferroni post hoc analysis. Cav-1 43 and Cav-1 37 had no effect on actin levels. B, representative blot showing the effects of the siRNAs on total and biotinylated EAAC1. Total and EAAC1 cell surface expression was calculated as a percentage of that observed in cells transfected with control siRNA. The amount of EAAC1 immunoreactivity in the lysate was not significantly different, but biotinylated EAAC1 was significantly decreased. Double asterisk indicates p < 0.01 compared with cells transfected with control siRNA by ANOVA using a Bonferroni post hoc analysis. In cells transfected with control siRNA, the amount of biotinylated actin was 3 ± 2% (mean ± S.E.), and this was not different between groups. D, data were normalized to total EAAC1 and expressed as a percentage of the immunoreactivity observed in control cells. Asterisk indicates a p < 0.05 compared with cells transfected with control siRNA by ANOVA using a Bonferroni post hoc analysis. E, samples were also analyzed for transferrin receptor immunoreactivity. The upper panel is a Western blot of biotinylated transferrin receptor (TIR) and the lower panel is the percentage of total transferrin receptor biotinylated expressed as a percentage of control. Data are the mean ± S.E. of five independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Effects of siRNA-mediated Knock-down of Caveolin-1 on EAAC1 Cell Surface Expression—One caveat associated with the use of dominant-negative mutants is the possibility that overexpression may result in nonspecific effects. Therefore, to complement this approach, siRNAs were used to knock-down caveolin-1 expression. Both siRNAs 37 and 43 reduced caveolin-1 expression by ∼60% (Fig. 4A), but these siRNAs did not reduce actin expression (Fig. 4B) nor did they affect total EAAC1 expression (Fig. 4C). However, both siRNAs significantly reduced the amount of biotinylated EAAC1 to ∼70% of that observed in control cells. The amount of biotinylated transporter was also normalized to total transporter expression and expressed as a percentage of that observed in cells transfected with a control siRNA. Both siRNAs directed against caveolin-1 significantly reduced the percentage of cell surface EAAC1 (Fig. 4D). To test the specificity of this effect, cell surface expression of the transferrin receptor was also examined in the same samples. Knock-down of caveolin-1 expression had no significant effect on the percentage of transferrin receptor found at the cell surface in these samples.

Kinetics of EAAC1 Delivery in Primary CNS Cultures Derived from Caveolin-1 Null Mice—To determine whether these effects generalize to a cellular system that may more closely recapitulate that observed in vivo, steady state levels of biotinylated EAAC1 were compared in primary cultures (neurons on a monolayer of astrocytes) derived from wild-type and caveolin-1 knock-out mice. In every set of cultures, expression of caveolin-1 was examined and the caveolin knock-out mice express no immu-
There was no significant difference between groups.

Seven independent experiments each conducted in duplicate (mean type and knock-out mice. The activity in the lysate and biotinylated fractions of cultures prepared from wild-type and knock-out mice. The upper panel is a representative Western blot of EAAC1 and actin immunoreactivity in the lysate and biotinylated fractions of cultures prepared from wild type and knock-out mice. The lower panel summarizes the results obtained in seven independent experiments each conducted in duplicate (mean ± S.E.). There was no significant difference between groups. C, analysis of delivery of EAAC1 as described under “Experimental Procedures.” Representative Western blots showing EAAC1 and actin immunoreactivity in both the cell lysate and biotinylated fraction. D, top panel, analysis of the increase in biotinylated EAAC1 as described in the legend to Fig. 3. The change in biotinylated myc-EAAC1 was linear to 30 min with mean r² values of 0.98 ± 0.01 (wild-type) and 0.95 ± 0.03 (caveolin-1 knock-out). Bottom panel, comparison of rates of delivery of EAAC1. Asterisk indicates a p < 0.05 compared with WT by Student’s t test. E, representative Western blots showing transferrin receptor (TfR) and actin immunoreactivity in the cell lysate and biotinylated fraction of cultures obtained from wild-type and caveolin-1 knock-out mice. F, top panel, analysis of delivery of transferrin receptor as a function of time. The change in biotinylated transferrin receptor was not linear beyond 15 min. Bottom panel, summary of the rates of transferrin receptor delivery. Data presented in D and F are the mean ± S.E. of seven independent experiments.

FIGURE 5. Cell surface expression and delivery of EAAC1 in primary CNS cultures derived from control and caveolin-1 knock-out (KO) mice. A, representative Western blots of caveolin-1, EAAC1, and actin expression in duplicate samples of primary cultures from wild-type and knock-out mice. B, the upper panel is a representative Western blot of EAAC1 and actin immunoreactivity in the lysate and biotinylated fractions of cultures prepared from wild type and knock-out mice. The lower panel summarizes the results obtained in seven independent experiments each conducted in duplicate (mean ± S.E.). There was no significant difference between groups. C, analysis of delivery of EAAC1 as described under “Experimental Procedures.” Representative Western blots showing EAAC1 and actin immunoreactivity in both the cell lysate and biotinylated fraction. D, top panel, analysis of the increase in biotinylated EAAC1 as described in the legend to Fig. 3. The change in biotinylated myc-EAAC1 was linear to 30 min with mean r² values of 0.98 ± 0.01 (wild-type) and 0.95 ± 0.03 (caveolin-1 knock-out). Bottom panel, comparison of rates of delivery of EAAC1. Asterisk indicates a p < 0.05 compared with WT by Student’s t test. E, representative Western blots showing transferrin receptor (TfR) and actin immunoreactivity in the cell lysate and biotinylated fraction of cultures obtained from wild-type and caveolin-1 knock-out mice. F, top panel, analysis of delivery of transferrin receptor as a function of time. The change in biotinylated transferrin receptor was not linear beyond 15 min. Bottom panel, summary of the rates of transferrin receptor delivery. Data presented in D and F are the mean ± S.E. of seven independent experiments.

Effects of Caveolin-1 Knock-out on the Kinetics of EAAC1 Endocytosis—To determine whether caveolin-1 knock-out also changes transporter endocytosis, EAAC1 internalization was compared in primary cultures derived from wild-type and caveolin-1 knock-out mice (Fig. 6). In cultures from wild-type and
Caveolin interacts with several plasma membrane proteins and internalization of caveolin-1 function results in reduced EAAC1 mice. Suggesting that as was observed with Cav-1 S80E, significantly slower in cultures prepared from caveolin-1 knock-out mice. The half-life of EAAC1 at the cell surface was somewhat lower in cultures prepared from Cav-1 S80E expressing C6 cells, total immunoreactivity (T), not re-warmed cells (0 min), and re-warmed for 5, 10, or 15 min at 37 °C. The lower panel is a representative Western blot of the total immunoreactivity. In these experiments, the percentage of un-stripped at time 0 min was 24 ± 1% in cultures from WT mice and 27 ± 3% in cultures prepared from KO mice. The net increase in inaccessible biotinylated myc-EAAC1 was expressed as a percentage of the total pool of transporter that could be stripped, and these data were fit to a first-order decay process. C, half-lives of internalization of EAAC1; these values (mean ± S.E.) were calculated from the rates obtained in five independent experiments. Asterisk indicates a p < 0.05 compared with WT by Student’s t test.

FIGURE 7. Caveolin-1, caveolin-2, and Cav-1 S80E formed immunoprecipitable complexes with EAAC1. A, immune complexes were isolated from C6 cells and probed for caveolin-1 or caveolin-2 immunoreactivity, arrows show the location of caveolin-1 and -2 immunoreactivity (Western blot (WB), Cav-1, or Cav-2). As positive control for caveolin immunoreactivity 5 μg of cell lysate were loaded (Lysate). B, preabsorbed or non-preabsorbed antibody was incubated overnight with C6 cells lysate. Immune complexes were assayed for caveolin-1 immunoreactivity and re-probed for EAAC1 immunoreactivity. C, the immunoprecipitates were obtained using an anti-EAAC1 antibody and not an anti-caveolin-1 or anti-caveolin-2 antibodies. As shown in Fig. 7A, caveolin-1 immunoreactivity was detected in immunoprecipitates obtained using an anti-EAAC1 antibody, and only a faint band was detected after immunoprecipitation with a control antibody (rabbit IgG). To further test the specificity of the immunoprecipitation procedure, the anti-EAAC1 antibody was pre-absorbed with the corresponding antigenic peptide, and no caveolin-1 immunoreactivity was observed (Fig. 7B). In addition, immunoprecipitation of EAAC1 with an anti-EAAC1 antibody from a different source (kindly provided by Dr. Jeffrey Rothstein, Johns Hopkins University) produced similar results (data not shown, n = 2). To determine whether these complexes contain another member of the caveolin family, immunoprecipitates were probed for caveolin-2. Caveolin-2 immunoreactivity was observed in immunoprecipitates obtained using an anti-EAAC1 antibody and not in control immunoprecipitates obtained with control IgG (Fig. 7A). These observations suggested that EAAC1 either directly or indirectly interacts with caveolin-1 and caveolin-2.

The dominant-negative variant of Cav-1 S80E is retained in intracellular compartments (44). If the trafficking of EAAC1 is facilitated by an interaction between EAAC1 and caveolin-1, it is possible that Cav-1 S80E reduces the levels of myc-EAAC1 at the plasma membrane by “retaining” the transporter in an intracellular compartment. To test if Cav-1 S80E interacts with EAAC1, HEK-293T cells were co-transfected with EAAC1 and Cav-1 WT or Cav-1 S80E cDNAs. In these cells EAAC1 immunoreactivity migrates as both a terminally glycosylated protein of ~70 kDa (and multimers thereof) and as an immature partially glycosylated protein (for discussion, see Ref. 49). EAAC1 immunoreactivity was detected in immuno-

FIGURE 6. Endocytosis of EAAC1 is slower in primary cultures derived from caveolin-1 null mice. A, representative Western blots showing the amount of biotinylated EAAC1 immunoreactivity in primary cultured cells, total immunoreactivity (T), not re-warmed cells (0 min), and re-warmed for 5, 10, or 15 min at 37 °C. The lower panel is a representative Western blot of the total immunoreactivity. In these experiments, the percentage of un-stripped at time 0 min was 24 ± 1% in cultures from WT mice and 27 ± 3% in cultures prepared form KO mice. B, the net increase in inaccessible biotinylated myc-EAAC1 was expressed as a percentage of the total pool of transporter that could be stripped, and these data were fit to a first-order decay process. C, half-lives of internalization of EAAC1; these values (mean ± S.E.) were calculated from the rates obtained in five independent experiments. Asterisk indicates a p < 0.05 compared with WT by Student’s t test.
precipitates obtained using an anti-caveolin-1 antibody from HEK-293T cells expressing Cav-1 WT or Cav-1 S80E (Fig. 7C). The predominant immunoreactivity observed in the immunoprecipitates represents immature EAAC1, suggesting that the interaction may in part develop during the posttranslational processing of the transporter. These observations provide further evidence that caveolin-1 and EAAC1 interact and identify a possible mechanism by which Cav-1 S80E may slow EAAC1 trafficking.

Effect of PMA Treatment on the Formation of EAAC1-Caveolin Complexes—Activation of PKC results in a redistribution of EAAC1 to the cell surface in both C6 cells and primary cultured neurons (9, 10, 13). Because caveolin appears to be involved in EAAC1 redistribution to the cell surface, it is possible that EAAC1 redistribution to the plasma membrane may be paralleled by an increased interaction with caveolin. To test this, C6 cells were treated with PMA for varying periods of time followed by immunoprecipitation of EAAC1. PMA increased the amount of caveolin-1 and caveolin-2 immunoreactivity observed in EAAC1 immunoprecipitates with a maximal amount of caveolin immunoreactivity observed between 15 and 60 min of PKC activation (Fig. 8, A and B). This parallels the time course observed for PKC-dependent redistribution of EAAC1 to the plasma membrane (14). Activation of the PDGF receptor also increases EAAC1 cell surface expression (11). This raises the possibility that activation of PDGF receptors may also increase the EAAC1-caveolin interaction. C6 cells were treated with PDGF for 30 min, and the levels of caveolin-1 immunoreactivity were examined. In cells exposed to PDGF, caveolin-1 immunoreactivity in EAAC1 immunoprecipitates was greater than in control cells (Fig. 8C), but it was less than that observed after PMA stimulation. These observations suggest that the interaction between EAAC1 and caveolin may play a role in the redistribution of transporter molecules to the plasma membrane.

**DISCUSSION**

In the present study, we found that co-expression of Cav-1 S80E or siRNA-mediated knock-down of caveolin-1 reduced the percentage of EAAC1 at the cell surface. Impaired caveolin-1 function by co-expressing Cav-1 S80E or knock-out of caveolin-1 slowed the delivery and endocytosis of EAAC1. siRNA-mediated knock-down of caveolin-1 had no effect on cell surface expression of transferrin receptor and knock-out of caveolin-1 did not slow the rate of delivery of transferrin receptor to the plasma membrane, suggesting that the effects on EAAC1 do not generalize to all recycling membrane proteins.

We also explored the possibility that trafficking of EAAC1 may depend on the formation of complexes between EAAC1 and caveolin-1. We found evidence for the formation of such complexes, and that the amount of caveolin immunoreactivity recovered in EAAC1 immunoprecipitates after PMA exposure parallels the redistribution of EAAC1 to the cell surface. We did not determine whether direct phosphorylation of EAAC1 is required to increase the amount of complex immunoprecipitated, and it is unclear if these effects are due to direct phosphorylation of caveolin (for review, see Ref. 23). We acknowledge that these studies simply show an association between effects on trafficking and formation of an immunoprecipitable complex. Future studies will be required to define the sites of interaction between caveolin-1 and EAAC1 to elucidate if the interaction is required for caveolin-dependent regulation of EAAC1 trafficking. In addition, it will be important to determine whether direct phosphorylation of either protein is required for this interaction.

Originally caveolae were described as plasma membrane invaginations thought to be involved in endocytic processes (for review, see Refs. 29–32). Since then caveolae have been implicated in the internalization of a number of diverse molecules (29–32, 50). More recent studies suggest that caveolin may have a more diverse array of functions, including the regulation of protein trafficking (22, 29, 48). Caveole-like vesicles appear to function as mobile “containers” between organelles and may be part of the machinery for the delivery of lipid rafts and associated proteins to the plasma membrane (51–53). Disruption of caveolin function promotes intracellular retention and accumulation of glycosylphosphatidylinositol-linked proteins, angiotensin II type 1 receptor and dysferlin (48, 54, 55). In addition, there is evidence that cholinergic and angiotensin II type 1 receptors are “escorted” by lipid rafts and/or caveolin through the exocytic pathway toward the cell surface (48, 56), suggesting that in some instances caveolin-1 may function as a “chaperone” required for the efficient delivery of proteins to the plasma membrane.

There is evidence that caveolin and/or lipid rafts may also regulate trafficking of plasma membrane transporters. In cells lacking caveolin, glucose transporter 4 is not redistributed to the plasma membrane after insulin stimulation, and glucose uptake is reduced. Apparently, glucose transporter 4 remains “trapped” in a perinuclear region of these cells (57). Disruption...
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of lipid rafts by cholesterol depletion decreases glutamate and serotonin transporter-mediated uptake (25, 58). Also, disruption of rafts blocks the PKC-dependent internalization of serotonin transporter (27, 58). However, the specific participation of caveolin has not been established.

Members of the soluble N-ethylmaleimide-sensitive attachment protein receptor (SNARE) family have been isolated as components of lipid raft fractions containing caveolin (59). Because the efficient fusion of vesicles is facilitated by SNAREs (for reviews, see Refs. 60 and 61) this observation suggests that: (i) raft association may allow SNARE clustering by favoring the formation of complexes between the vesicles and the membrane, and/or (ii) raft association may allow spatial segregation of proteins and protein complexes important for exocytosis (59). Two members of the SNARE family, syntaxin 1A and SNAP-23, regulate the cell surface expression of EAAC1 (39, 62, 63). This provides a possible mechanism for the involvement of lipid rafts and/or caveolin in the trafficking of neurotransmitter transporters, but the possible coupling between SNAREs and lipid rafts and/or caveolin remain to be investigated.

An additional function attributed to caveolae is the organization and compartmentalization of signal transduction pathways. Although it is unclear if caveolae and/or caveolin are necessary for the recruitment of transporters and signaling molecules to specific domains within the cell, our identification of EAAC1-caveolin complexes suggests this as a possibility. Some proteins like GTRAP3–18 and syntaxin 1A regulate maturation and trafficking of transporters (62–64). Others like TRAPP (27, 58). However, the specific participation of rafts blocks the PKC-dependent internalization of serotonin transporter (27, 58). Finally, proteins like the serum- and glucocorticoid-inducible kinase-1 and PKC

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