Characterization of an epilepsy-associated variant of the human Cl\(^{-}/\)HCO\(_3\)^{-} exchanger AE3

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Vilas GL, Johnson DE, Freund P, Casey JR. Characterization of an epilepsy-associated variant of the human Cl\(^{-}/\)HCO\(_3\)^{-} exchanger AE3. Am J Physiol Cell Physiol 297: C526–C536, 2009. First published July 15, 2009; doi:10.1152/ajpcell.00572.2008.—Anion exchanger 3 (AE3), expressed in the brain, heart, and retina, extrudes intracellular HCO\(_3\)^{-} in exchange for extracellular Cl\(^{-}\). The SLC4A3 gene encodes two variants of AE3, brain or full-length AE3 (AE3\(_{\text{fl}}\)) and cardiac AE3 (cAE3). Epilepsy is a heterogeneous group of disorders characterized by recurrent unprovoked seizures that affect about 50 million people worldwide. The AE3-A867D allele in humans has been associated with the development of IGE (IGE), which accounts for ∼30% of all epilepsies. To examine the molecular basis for the association of the A867D allele with IGE, we characterized wild-type (WT) and AE3\(_{\text{fl}}\)-A867D in transfected human embryonic kidney (HEK)-293 cells. AE3\(_{\text{fl}}\)-A867D had significantly reduced transport activity relative to WT (54 ± 4%, P < 0.01). Differences in expression levels or the degree of protein trafficking to the plasma membrane did not account for the defect of AE3\(_{\text{fl}}\)-A867D. Treatment with 8-bromo-cAMP (8-Br-cAMP) increased Cl\(^{-}/\)HCO\(_3\)^{-} exchange activity of WT and AE3\(_{\text{fl}}\)-A867D to a similar degree, which was abolished by preincubation with the protein kinase A (PKA)-specific inhibitor H89. This indicates that PKA regulates WT and AE3\(_{\text{fl}}\)-A867D to a similar degree, which was abolished by preincubation with the protein kinase A (PKA)–specific inhibitor H89.

Although AE3 is differentially expressed in excitatory tissues, the two isoforms have different distributions (30). AE3\(_{\text{fl}}\) and cAE3 are expressed in the heart and retina, whereas in the brain only AE3\(_{\text{fl}}\) is expressed (46). In the brain, where neuronal activity generates rapid and significant pH\(_{i}\) changes, AE3\(_{\text{fl}}\) activity may be important for the removal of excessive intracellular HCO\(_3\)^{-}, thus contributing to the maintenance of normal neuronal and glial function (13, 30, 44). Neurotransmitter receptors for γ-aminobutyric acid (GABA) and glycine have intrinsic Cl\(^{-}\) channel activity (25). Since the degree and polarity of the response is determined by the intracellular levels of Cl\(^{-}\), it has been suggested that AE3 is involved in the modulation of this response through its concentrative Cl\(^{-}\) activity (6).

Epilepsy is a heterogeneous group of seizure disorders that affect about 50 million people worldwide (43). Idiopathic generalized epilepsy (IGE) accounts for ∼30% of all epilepsies (45). Patients affected with IGE manifest symptoms between early childhood and adolescence. IGE is characterized by recurrent and unprovoked generalized seizures that are apparently unrelated to brain lesions and/or metabolic disorders (3, 7). Genetic studies of monogenic idiopathic epilepsies identified mutations in some central nervous system ion channels (KCNJ10, KCNJ3, KCNQ2/KCNQ3, CLCN2, GABRG2, GABRA1, SCN1B, and SCN1A), suggesting that IGE presents a complex pattern of inheritance and that several genetic factors contribute to the predisposition to generalized seizures (20, 34).

The occurrence of the AE3-A867D allele in humans is associated with the development of IGE (40), yet the effect of the A867D variant on AE3 function has not been tested. AE3 knockout mice, however, display a reduced threshold for chemically induced seizures (23). Together this suggests that defects in AE3 activity may cause seizures due to abnormal regulation of neuronal pH\(_{i}\) and cell volume.

In this work we evaluated the functional and physiological parameters of wild-type (WT) and human AE3\(_{\text{fl}}\)-A867D in transiently transfected human embryonic kidney (HEK)-293 cells to investigate the potential role of AE3\(_{\text{fl}}\)-A867D in the development of IGE. Only AE3\(_{\text{fl}}\) has been studied here, therefore, for the sake of simplicity, “AE3” as used here will imply the AE3\(_{\text{fl}}\) variant. We found that WT and AE3-A867D proteins had a similar subcellular localization and were processed equally to the plasma membrane. AE3-A867D, however, had significantly reduced transport activity when compared with WT. Incubation of transiently transfected HEK-293 cells with...
protein kinase A (PKA) agonists and inhibitors showed that AE3\textsubscript{a} transport activity was regulated by this kinase. When taken together, the results indicate that the decreased transport activity of AE3-A867D may cause changes in cell volume and abnormal pHi and Cl\textsuperscript{−} levels. In the brain these alterations may promote neuron hyperexcitability and contribute to the generation of epileptic seizures.

MATERIALS AND METHODS

\textbf{Materials}. Eukaryotic-expression construct for human AE3 cDNA was a gift from Dr. Seth Alper (Beth Israel Deaconess Medical Center, Boston, MA). Dual-emission, pH-sensitive green fluorescent protein cDNA (de4gfp/peGFP-N1) was a gift from Dr. Jim Remington (University of Oregon). cDNA encoding GPI-eGFP, an enhanced green fluorescent protein, containing the signal sequence of lactase-phlorizin hydrodase and a consensus N-glycosylation site fused to the glycosylphosphatidylinositol (GPI)-attachment signal of the protein function-associated-antigen 3 (28), was from Dr. Todd Alexander (University of Alberta). Oligonucleotides were from Integrated DNA Technologies (Corvalis, IA). Restriction enzymes were from New England Biolabs (Ipswich, MA). PfX DNA polymerase, Dulbecco’s modified Eagle’s medium (DMEM), neurobasal medium, fetal bovine serum, calf serum, penicillin-streptomycin-glutamine, and BCECF-AM were from Invitrogen (Carlsbad, CA). Cell culture dishes were from Corning (Corning, NY). Glass coverslips were from Fisher Scientific (Ottawa, ON, Canada). Complete protease inhibitor was from Roche Applied Science (Indianapolis, IN). BCA Protein Assay Kit and immobilized streptavidin resin were from Pierce (Rockford, IL). H89, 8-bromo-cAMP (8-Br-cAMP), nigericin, and poly-l-Lysine were from Sigma-Aldrich (Oakville, ON, Canada). Mouse anti-glycosylated-phosphatidyl inositol (GPI)-attachment signal of lymphocyte-function-associated-antigen 3 (28) was a gift from Dr. Todd Alexander (University of Alberta). Anti-mouse IgG and protein A Sepharose CL-4B were from GE Healthcare Bio-Sciences (Piscataway, NJ). HRP-conjugated donkey anti-rabbit IgG were from Sigma-Aldrich (Oakville, ON, Canada). Complete Protease Inhibitor. Protein content was determined using the BCA method.

\textbf{Photometric measurements of cytosolic pH}. HEK-293 cells transiently transfected with WT AE3, AE3-A867D, deGFP4, or pcDNA constructs or hippocampal neurons from WT or ae3\textsuperscript{−/−} mice were grown on 25-mm circular poly-l-lysine-coated glass coverslips. Coverslips were rinsed with 4°C PBS (in mM: 140 NaCl, 3 KCl, 6.5 Na\textsubscript{2}HPO\textsubscript{4}, 1.5 KH\textsubscript{2}PO\textsubscript{4}, pH 7.4), lyzed in IPB buffer (1% NP40, 5 mM EDTA, 0.15 M NaCl, 0.5% deoxycholate, and 10 mM Tris-HCl, pH 7.5), containing Complete Protease Inhibitor. Protein content was determined using the BCA method.

\textbf{Tissue culture}. WT AE3, AE3-A867D, or pcDNA were expressed alone or coexpressed with deGFP4 in a 4:1 molar ratio, by transient transfection of HEK-293 cells, using the calcium phosphate method (38). Cells were grown at 37°C in an air-CO\textsubscript{2} (19:1) environment in DMEM, supplemented with 5% (vol/vol) fetal bovine serum, 5% (vol/vol) calf serum, and 1% (vol/vol) penicillin-streptomycin-glu-tamine. All experiments involving transfected cells were carried out 40 to 48 h posttransfection.

\textbf{Isolation of hippocampal neurons}. Hippocampi were dissected from the brains of 15.5 d WT and slc4a3\textsuperscript{−/−} mice mouse embryos (19 WT and 14 ae3\textsuperscript{−/−}) (17). slc4a3\textsuperscript{−/−} mice, whose characterization and method of genotyping have been previously reported (1), were obtained from Dr. Gary Shull (University of Cincinnati). Samples were incubated for 25 min at 37°C in Hanks’ buffered saline solution (in mM: 5.3 KCl, 137.9 NaCl, 4.6 d-glucose, 0.4 KH\textsubscript{2}PO\textsubscript{4}, 0.3 Na\textsubscript{2}HPO\textsubscript{4}, and 4.2 NaHCO\textsubscript{3}, pH 7.3), containing 0.5% (vol/vol) trypsin. After incubation neurobasal medium, supplemented with B27 supplement, 200 mM l-glutamine and 10,000 U/ml penicillin-streptomycin were added, and samples were mixed for 1–2 min by inversion. Samples were centrifuged at 600 g for 5 min. Supernatant was aspirated and the samples were further triturated 10 times with a Pasteur pipette in fresh neurobasal medium. Samples were left to settle for 5 min, and the supernatant was transferred to a new tube. Samples were centrifuged at 150 g for 5 min. Pellets were resuspended in 4 ml medium. Cells were counted, and ~1 × 10\textsuperscript{6} cells/ml were plated onto 25-mm poly-l-lysine-coated coverslips. Cultures were incubated at 37°C in an air-CO\textsubscript{2} (19:1) environment and assayed for Cl\textsuperscript{−}/HCO\textsubscript{3} exchange activity 16–26 h after isolation.

\textbf{Fluorimeter-based measurements of cytosolic pH}. HEK-293 cells transiently transfected with WT AE3, AE3-A867D, deGFP4, or pcDNA constructs or hippocampal neurons from WT or ae3\textsuperscript{−/−} mice were grown on 25-mm circular poly-l-lysine-coated glass coverslips. Coverslips were rinsed with 4°C PBS (in mM: 140 NaCl, 3 KCl, 6.5 Na\textsubscript{2}HPO\textsubscript{4}, 1.5 KH\textsubscript{2}PO\textsubscript{4}, pH 7.4), lyzed in IPB buffer (1% NP40, 5 mM EDTA, 0.15 M NaCl, 0.5% deoxycholate, and 10 mM Tris-HCl, pH 7.5), containing Complete Protease Inhibitor. Protein content was determined using the BCA method.
quantified on immunoblots subjected to densitometry. Data are presented as percentage of WT AE3 and corrected for background exchange activity of empty vector-transfected cells.

Coverslips containing hippocampal neurons from WT or slc4a3−/− embryonic mice previously incubated with 3 μM BCECF-AM were mounted on the microscope stage, perfused, and analyzed as described above. Approximately 20 cells were selected for pH measurements.

Measurement of intrinsic buffer capacity and proton flux. Intracellular buffer capacity measurements were made by the ammonium chloride pulse method (33). Briefly, HEK-293 cells were transiently transfected with WT or A867D cDNA AE3 as described above, and 2 days posttransfection the cells were loaded with BCECF-AM. Coverslips were mounted in a fluorescence cuvette and allowed to equilibrate in Ringer buffer without NaHCO3. Cells were consecutively perfused for 200 s with NaHCO3-free Ringer buffer containing 20, 10, 5, 1, and 0 mM NH4Cl. Fluorescence changes were recorded and the data were converted into pHi using the nigericin-high potassium method as described above. The NH4Cl intracellular concentration ([NH4Cl]i) was calculated using the Henderson-Hasselbalch equation, and the intrinsic buffer capacity (βi) was then calculated as Δ[NH4Cl]/ΔpHi (42, 47). Total buffer capacity of the cells (βtotal) was calculated as: βtotal = βi + βeo, where βeo = 2.3 [HCO3−] (33). Proton equivalent flux (JpH) was calculated as: JpH = (ΔpHi/Δt) × βtotal.

Immunoblot analysis. Samples were prepared in 2× SDS-PAGE sample buffer [10% (vol/vol) glycerol, 2% (wt/vol) SDS, 0.5% (wt/vol) bromophenol blue, 75 mM Tris, pH 6.8], containing complete protease inhibitor. Before analysis, samples were made to 1% (vol/vol) 2-mercaptoethanol and heated for 4 min at 65°C, and insoluble material was removed by centrifugation at 16,000 g for 10 min. Samples were then resolved by SDS-PAGE on 7.5% (wt/vol) acrylamide gels (31). Proteins were electrotransferred onto Immobilon-P PVDF membranes (Millipore, MA) for 1 h at a constant current of 400 mA. After transfer, membranes were rinsed in TBS (0.15 M NaCl, 50 mM Tris-HCl, pH 7.5) and incubated with 5% (wt/vol) skim milk TBS-T (0.1% (vol/vol) Tween-20, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.5) for 1 h at room temperature with gentle rocking to block nonspecific binding. Membranes were then incubated for 16 h at 4°C with gentle rocking in the presence of either rabbit anti-human AE3ct, mouse anti-GAPDH at a 1:2,500 or 1:2,000 dilution in 5% skim milk TBS-T, respectively. After successive washes with TBS and TBS-T, the membranes were incubated with a 1:5,000 dilution of the appropriate HRP-conjugated secondary antibodies in 5% skim milk TBS-T for 1 h at room temperature and further washed with TBS and TBS-T. Proteins were detected using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer) and visualized using a Kodak Image Station 440CF (Kodak, NY). Quantitative densitometric analyses were performed using Kodak Molecular Imaging Software v4.0.3 (Kodak, NY).

Cell surface processing assay. HEK-293 cells were transiently transfected with cDNAs encoding WT, AE3-A867D, or pcDNA 3.1 (empty vector) as described above. Forty to forty eight hours posttransfection, cells were rinsed with 4°C PBS, washed with 4°C borate buffer (mM: 154 NaCl, 7.2 KCl, 1.8 CaCl2, and 10 boric acid, pH 9.0) and then incubated for 30 min at 4°C in borate buffer containing Sulfo-NHS-SS-Biotin (0.5 mg/ml, Pierce, IL). After being washed three times with 4°C quenching buffer (152 mM glycine, 25 mM Tris, pH 8.3), cells were solubilized 20 min on ice in 500 μl of IPB, containing complete protease inhibitor. Cell lysates were centrifuged for 20 min at 13,200 g and the supernatants recovered. For each sample, the amount of the supernatant was retained for later SDS-PAGE analysis (total protein, T). The remaining half of the supernatant was combined with 50 μl of a 50% slurry of immobilized streptavidin resin in PBS and incubated 16 h at 4°C with gentle rotation. Samples were centrifuged for 2 min at 8,000 g, and the supernatant was collected (unbound protein, U). The T and U fractions of each sample were analyzed by SDS-PAGE and immunoblotting as described above. After densitometric quantitation of the corresponding bands, the percentage of biotinylated protein was calculated as (T – U)/T × 100%.

Confocal microscopy. Cells grown on 22 × 22 mm poly-l-lysine-coated coverslips were individually transfected with WT, AE3-A867D cDNAs, or empty vector or cotransfected with GFP-eGFP as described above. Two days posttransfection cells were washed twice with PBS, fixed with 3.5% paraformaldehyde, 1 mM CaCl2, and 1 mM MgCl2 in PBS, pH 7.4, for 20 min, washed twice with PBS, and then quenched with 50 mM NH4Cl for 10 min. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 1 min at room temperature, followed by a 30-min block with 0.2% (wt/vol) gelatin in PBS. After blocking was completed, coverslips were incubated for 1 h in a humidified chamber in the presence of goat anti-rabbit IgG conjugated with Alexa Fluor 594 and chicken anti-mouse IgG conjugated with Alexa Fluor 488 at 1:1,000 dilutions in 0.2% (wt/vol) gelatin in PBS, respectively. After three washes with 0.2% (wt/vol) gelatin in PBS, coverslips were further incubated for 1 h in a dark humidified chamber in the presence of goat anti-rabbit IgG conjugated with Alexa Fluor 594 and chicken anti-mouse IgG conjugated with Alexa Fluor 488 at 1:1,000 dilutions in 0.2% (wt/vol) gelatin in PBS to detect AE3 and calnexin, respectively. Finally, coverslips were washed three times with 0.2% (wt/vol) gelatin in PBS, rinsed twice with PBS, and mounted in Prolong Antifade Gold Solution containing the DNA-specific fluorescent dye 4′,6-diamidino-2-phenylindole. Images were obtained with a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss Micro-Imaging), mounted on an Axiovert 100M controller with a ×63 (numerical aperture 1.4) lens.

Colocalization studies. Confocal images, obtained as described above, were analyzed with Volocity software (Improvision, Waltham, MA).

Statistical analysis. Analysis was performed using Prism software (Graphpad). Groups were compared with one-way ANOVA and paired t-test with P < 0.05 considered significant.

RESULTS

The AE3 A867D sequence variant is associated with IGE, yet the functional effects of this change, whether it is a polymorphism or a functional mutation, have not been assessed. To determine the effects of this mutation, we compared the Cl−/HCO3− exchange activity of WT and mutant human AE3. HEK-293 cells were transiently transfected with either WT or AE3-A867D cDNA and subjected to Cl−/HCO3− exchange assays. Cells were loaded with the pH-sensitive dye BCECF-FM and alternatively perfused with Cl−-containing and Cl−-free (where equimolar membrane impermeant sodium gluconate replaces NaCl) buffers in a fluorescence cuvette (47). When perfused with Cl−-free buffer, AE3 mediates the efflux of cytoplasmic Cl− in exchange for extracellular HCO3−, which increases pHi, as measured by an increase in fluorescence. Figure 1A illustrates the typical anion-exchange data obtained for empty vector AE3-A867D and WT AE3. Steadystate pHi in Cl−-containing medium was 7.19 ± 0.03, 7.20 ± 0.004, and 7.20 ± 0.03 pH units for vector, AE3-A867D, and WT AE3, respectively (Supplemental Fig. 1A). Transport rates were determined from the initial alkalization rate upon switching from Cl−-containing to Cl−-free medium. Intrinsic buffer capacity (βi) of vector A867D and WT AE3-transfected HEK-293 cells at pH 7.20 were 10 ± 1, 10 ± 4, and 10 ± 1 mM/pH, respectively (Supplemental Fig. 1B). Flux of proton equivalents (in units of mM·min−1) was then calculated as dpH/dt/βtotal. Cl−/HCO3− exchange rate of AE3-A867D transfected cells was significantly slower (P < 0.05) than WT AE3-transfected cells (5 ± 1 and 11 ± 2 mM/min, respec-
AE3-A867D had a significantly lower relative Cl\(^{-}\) exchange activity (47 ± 13%, P < 0.05) when compared with WT AE3 (Fig. 1D).

AE3-A867D transport activity was also assessed in a small group of cells monitored on a microscope stage. HEK-293 cells were transiently cotransfected with WT AE3, AE3-A867D, or pcDNA3.1 and cDNA encoding the dual-emission, pH-sensitive green fluorescent protein deGFP4 (21) at a 4:1 molar ratio. Importantly, deGFP4 fluorescence is virtually Cl\(^{-}\) insensitive (26), which makes it ideal for monitoring pH\(_{i}\) changes associated with Cl\(^{-}/\)HCO\(_{3}^{-}\) exchange. Fluorescence of three to five transfected cells (identified by their green fluorescence) was monitored by photometry as the cells were perfused alternately with Cl\(^{-}\)-containing and Cl\(^{-}\)-free Ringer buffers, as described above. At the end of each experiment, fluorescence values were calibrated to pH using the nigericin-high potassium technique, and Cl\(^{-}/\)HCO\(_{3}^{-}\) exchange activity was calculated by the linear regression of the initial rate of pH\(_{i}\) change during perfusion with Cl\(^{-}\)-free buffer. C: cell lysates, prepared from cells used in Cl\(^{-}/\)HCO\(_{3}^{-}\) exchange assays, were subjected to immunoblotting and probed with AE3ct antibody, which recognizes the COOH-terminal tail of AE3 protein. D: transport activities corrected for activity of vector-transfected cells and normalized for AE3 protein expression. Values are expressed as percentage of WT AE3. Error bars represent SE (n ≥ 5). *Significant difference (P < 0.05) compared with WT AE3 exchange activity.
Fig. 2. Cl−/HCO3− exchange activity of WT and AE3-A867D measured by microscopic photometry. A: HEK-293 cells were transiently cotransfected with deGFP4 cDNA and WT AE3, AE3-A867D, or empty vector in a 1:4 molar ratio. deGFP4 fluorescence was monitored at λex = 400 nm and λem = 460 and 510 nm. Cells were perfused alternately with Cl−-containing (shaded bar) and Cl−-free (open bar) Ringer buffer. At the end of each experiment fluorescence values for pH-sensitive deGFP were calibrated to pH, via the nigericin-high potassium technique. Blue, red, and black traces represent results from WT AE3, AE3-A867D, and vector-transfected cells, respectively. B: mean Cl−/HCO3− exchange activity calculated by linear regression of the initial rate of pH change during perfusion with Cl−-free buffer. C: transport activities, corrected for activity of vector-transfected cells. Error bars represent SE (n = 3).

Mutations in membrane proteins often result in misfolding that may lead to decreased activity, impairment of biogenesis, endoplasmic reticulum retention, and abolished or decreased expression at its target membrane(s) (4, 22). Since anion-exchange assays only measure the activity of proteins at the plasma membrane, reduced cell surface expression levels of functional AE3 may result in an apparent lower anion exchange activity. To test whether the AE3-A867D mutation affects AE3 membrane processing, we quantified the amount of protein present at the cell surface by cell surface biotinylation assays of HEK-293 cells transiently transfected with empty vector, AE3-A867D, or WT AE3. Two days posttransfection the cells were labelled with the membrane-impermeant biotinylation reagent Sulfo-NHS-SS-biotin and then lysed with IPB buffer. After solubilization, half of the total lysate (T) was retained and the remainder was incubated with streptavidin-agarose resin and centrifuged to remove biotinylated protein. Equal volumes of biotin-free (U) and total (T) lysates were separated by SDS-PAGE. The presence of AE3 and the endogenous cytosolic protein GAPDH (negative control) was determined by immunoblot (Fig. 3A). Densitometric analysis revealed that 37 ± 5% of WT, 32 ± 2% of AE3-A867D, and 8 ± 2% of GAPDH were accessible to the biotinylation reagent, implying plasma membrane localization (Fig. 3B). The presence of a low level of biotinylated GAPDH may reflect the fact that some of the cells were damaged during the experiment therefore exposing cytoplasmic proteins to the labeling reagent. Thus the GAPDH plasma membrane localization value (8 ± 2%) represents the background value for the assay. The AE3 cell surface processing percentage presented in this work further confirms the values previously reported by our laboratory for this protein (47). The difference between WT and AE3-A867D cell surface processing was statistically insignificant, thus suggesting that the plasma membrane expression is similar for both proteins.
AE3 mutant in epilepsy

Fig. 3. Assay of cell surface localization of human WT and AE3-A867D. HEK-293 cells were transiently transfected with cDNA encoding either WT or AE3-A867D or empty vector. Cells were labeled with Sulfo-NHS-SS-biotin and lysed, and half of the lysate was incubated with streptavidin-agarose resin to remove biotinylated proteins. A: equal fractions of total lysate (T) and unbound supernatant fraction (U) were separated by SDS-PAGE. Immunoblots were probed with AE3ct anti-AE3 and anti-GAPDH antibodies. B: fraction of protein labeled by the membrane-impermeant biotinylation reagent (representing protein at the cell surface), as calculated by densitometry, is shown. Error bars represent SE (n = 6). t-Test revealed no significant difference (NS) between the two AE3 proteins.

To investigate further whether the A867D mutation affects AE3 biogenesis, we compared the subcellular localization of WT and AE3-A867D expressed in transiently transfected HEK-293 cells using confocal microscopy. WT and AE3-A867D had similar subcellular distribution patterns, with the majority of the protein present in the endoplasmic reticulum and some protein located at the plasma membrane, as judged by the extent of signal overlapping with the endoplasmic reticulum-specific marker calnexin (Fig. 4A) and the plasma membrane-targeted GPI-eGFP protein (Fig. 4B), respectively. Colocalization of the markers with AE3 was quantified, using Pearson’s coefficient (Fig. 4C). The degree of colocalization found for vector-alone transfected cells represents nonspecific signal and therefore the threshold above which there is significant overlap between the fluorescent signals. Framed in this way, both AE3 WT and AE3-A867D colocalize with calnexin and GPI-eGFP, indicating respectively localization of AE3 in the endoplasmic reticulum and at the plasma membrane. Colocalization analysis, however, revealed no significant difference in the localization of WT and AE3-A867D (Fig. 4C). Consistent with the biotinylation data (Fig. 3), these results indicate that at a steady state the majority of synthesized AE3 is present in the endoplasmic reticulum and that the mutation does not greatly affect the biogenesis and subcellular localization of exogenously expressed AE3-A867D. Furthermore, this result supports our previous finding that only a fraction of the synthesized protein reaches the plasma membrane. Taken together, the results presented so far indicate that differences in baseline pH, intrinsic buffer capacity, cell surface processing, and subcellular localization do not explain the reduced anion exchange activity of AE3-A867D.

The occurrence of the A867D AE3 allele in humans is associated with the development of IGE (40). Moreover, AE3 knockout mice display a reduced threshold for chemically induced seizures (23). Metabotropic glutamate receptors (mGluRs) are monomeric G protein-coupled receptors that contain a large NH2-terminal extracellular tail that harbors the glutamate binding site (14). These receptors transduce extracellular signals via the second messenger cAMP and participate in various adaptive and pathological neurological events, including synaptic plasticity, excitotoxicity, and the pathogenesis of epilepsy (11, 35). We thus considered whether cAMP-coupled signaling events might differentially regulate AE3 and the A867D variant. In support of the notion, protein kinase-specific eukaryotic protein phosphorylation prediction algorithms (8) indicated the presence of five PKA consensus phosphorylation motifs predicted to have ≥60% probability to act as a PKA site (data not shown) in the AE3 amino-terminal cytosolic domain.

We therefore characterized the effect of PKA on AE3 transport activity. Transport activity of HEK-293 cells transiently transfected with empty vector, AE3-A867D, or WT AE3 is significantly increased after incubation with the membrane-permeant cAMP analog and PKA agonist 8-Br-cAMP (100 μM for 10 min) and decreases when preincubated with the PKA inhibitor H89 (10 μM) for 5 min and then further incubated for 10 min in the presence of both H89 and 8-Br-cAMP (Fig. 5A).

To compare the effect that these treatments have on the transport activity of the two proteins, AE3-A867D and WT AE3 anion exchange activities were corrected for background Cl-/HCO3 exchange activity of empty vector-transfected cells. The resulting transport rates were normalized for the amount of AE3 expressed in the cells, which was detected by immunoblotting and quantified by densitometry. 8-Br-cAMP significantly increased both WT and AE3-A867D anion exchange activity by 26 ± 11% and 36 ± 9%, respectively, compared with corresponding untreated transfected cells (Fig. 5B).

Treatment with H89 not only abolished the stimulatory effects of 8-Br-cAMP but significantly decreased transport activity for both WT and AE3-A867D by 33 ± 13% and 19 ± 9%, respectively, when compared with untreated transfected cells, suggesting that there is basal cAMP-stimulation of AE3 transport activity in HEK-293 cells. As a control, anion exchange activity of transfected cells was measured after two successive Cl-/free buffer pulses in the absence of agonist, and
Fig. 4. Subcellular localization of WT AE3 and AE3-A867D. HEK-293 cells individually transfected with WT or AE3-A867D cDNAs or empty vector (A) or cotransfected with GPI-eGFP (B) were grown on glass coverslips and processed for confocal immunofluorescence microscopy. AE3 was detected using rabbit anti-AE3 antibody and goat anti-rabbit IgG conjugated with Alexa Fluor 594 (red). Endoplasmic reticulum (ER) marker calnexin (CNX) was detected with a mouse anti-CNX monoclonal antibody and chicken anti-mouse IgG conjugated with Alexa Fluor 488 (green). Green fluorescence of the plasma membrane marker GPI-eGFP was detected directly. Nuclei were detected with DAPI (blue), as indicated. The merged images are presented in the far right-hand column. Scale bars represent 10 μm. C: Pearson’s linear correlation coefficient overlap between fluorescence signals from ER marker CNX and plasma membrane-associated GPI-eGFP with WT (blue bars) and AE3-A867D (red bars) was measured using Volocity software. Black bars represent non-specific values calculated for vector-alone transfected cells. Error bars represent SE (n ≥ 6). t-Test revealed no significant difference in the degree of colocalization between the two AE3 proteins and the markers.
no significant differences were found in the two rates (100 ± 4% vs. 110 ± 20% for the first and second pulses, respectively).

Finally, we investigated whether the stimulation of transport activity observed upon treatment with 8-Br-cAMP was associated with an increase in the cell surface expression of AE3. HEK-293 cells, transiently transfected with AE3-A867D or WT AE3, were incubated with vehicle or 100 μM of the PKA agonist 8-Br-cAMP for 10 min, and the amount of protein present at the cell surface was quantified by cell surface biotinylation assays. Treatment with 8-Br-cAMP did not significantly increase the amount of either AE3-A867D or WT AE3 present at the plasma membrane (Supplemental Fig. 3), suggesting that cAMP does not alter AE3 activity through insertion of vesicular-localized AE3 into the plasma membrane. These results indicate that both WT and AE3-A867D transport activities increase similarly when intracellular cAMP levels are increased and that PKA is involved in this activation.

Taken together, the results presented here indicate that AE3-A867D has about half the Cl-/HCO3- exchange activity of WT AE3. Differences in cell surface processing, subcellular localization, and sensitivity to cAMP-coupled signaling pathways do not explain the decreased activity of AE3-A867D.

DISCUSSION

In this work we examined the transport activity, subcellular localization, and PKA-mediated regulation of AE3-A867D, an allele associated with development of IGE in humans (40). The results presented here reveal that the AE3-A867D allele represents a functional mutation, which results in a reduction in transport activity by about one-half relative to WT AE3. Decreased Cl-/HCO3- exchange activity of AE3-A867D is not caused by defects in protein expression level, cell surface processing, or subcellular localization, as assessed respectively by quantitative immunoblots, cell surface biotinylation assays, and confocal immunofluorescence. AE3 transport activity was increased by PKA, as shown by the stimulatory effects of 8-Br-cAMP, which were suppressed by the PKA inhibitor H89. The similar effect of PKA on WT and AE3-A867D indicates that differences in regulation by the cAMP-coupled signaling pathway do not explain the association of the allele with IGE seizures. We conclude that reduction of AE3 Cl-/HCO3- exchange activity by 50% is sufficient to promote increased susceptibility to epileptic seizures in humans.

In an attempt to understand the role that AE3 plays in seizure sensitivity in vivo, we measured the Cl-/HCO3- exchange activity of mixed hippocampal neuron-glia cultures isolated from WT and ae3−/− mice at developmental stage E15.5. We found no significant differences in either transport activity or baseline pHc, consistent with previous measurements of rat fetal hippocampal neurons, which did not display active Cl-/HCO3- exchange (37). Thus, whereas AE3 may have an important role in adult brain, the role is not manifested in fetal brain cells. These data suggest that despite the presence of AE3 message in fetal brain cells, in which the AE3-expressing cells were a population of cells, in which the AE3-expressing cells were a subpopulation whose activity could not be detected on the backdrop of other cells. Alternatively, AE3 expression may have been downregulated during the 16-h interval between the time the cells were isolated from the mice and the time Cl-/HCO3- exchange assays were performed. This time period was, unfortunately, the minimum period required to allow the cells to set down on the tissue culture dishes.

Humans carrying the A867D allele would likely be heterozygous. Since AE3-A867D has half the activity of WT
AE3, 75% of AE3 Cl⁻/HCO₃⁻ exchange activity from AE3 should remain. Thus the increase of epilepsy associated with AE3-A867D arises from only an expected loss of only 25% of AE3 transport activity. Is any insight available from ae3⁺/⁻ mice? Despite the finding that AE3 is the major Cl⁻/HCO₃⁻ exchanger in adult mice brain (23), ae3⁻/⁻ and ae3⁺/⁻ mice were fertile, developed normally, and were indistinguishably behaviorally and anatomically from their ae3⁺/⁺ littermates (1, 23). Closer examination revealed no obvious morphological changes or degeneration in the brain of the ae3⁺/⁻ animals (23). Disruption of the slc4a3 gene in these animals, however, resulted in higher sensitivity to seizure-inducing agents, inner retinal defects, and late onset photoreceptor death (1, 23). The ae3⁺/⁻ mice had more than a twofold reduction in AE3 protein expression in their retinas (1), suggesting that loss of one allele in some way affects accumulation of AE3 by the unaffected allele, or that cells expressing AE3 protein decrease in abundance. Unfortunately, there has been no report of the sensitivity of ae3⁺/⁻ mice to seizures.

Interestingly, in AE3-A867D, the mutated amino acid residue is present in the third extracellular loop of the protein, located between transmembrane segments 5 and 6 on the basis of sequence alignment with AE1, whose topology has been extensively studied (52). On the basis of this location, residues in this loop may interact with AE3 substrates on route to the plasma membrane to regulate activity. Computational studies predicted five Ser-containing sequences likely to be phosphorylated by PKA (phosphorylation probability ≥60%). Among these, a site encompassing Ser 553 was predicted to be phospho-rylated by PKA with the second highest degree of confidence (78%). PKA activity is dependent on the level of cAMP in the cell. Interestingly, an important group of neuronal receptors, the metabotropic glutamate receptors (mGluRs) involved in the pathogenesis of epilepsy, are G protein-coupled receptors and transduce extracellular cues via the second messenger cAMP (11, 35). We therefore studied whether the activity of both WT and AE3-A867D was modulated by PKA. The results obtained using PKA agonists and inhibitors on transiently transfected cells indicate that the activity of both AE3 isoforms is significantly increased by PKA and decreased in the presence of the PKA-specific inhibitor H89. These experiments indicate that the activity of AE3 is modulated by intracellular cAMP levels, in line with observations that PKA regulates the activities of the Na⁺-dependent and Na⁺-independent Cl⁻/HCO₃⁻ exchangers in adult rat CA1 neurons (9). This is, however, the first report on the regulation of AE3 by PKA or even cAMP. Interestingly, the extent of activation and inhibition of Cl⁻/HCO₃⁻ transport elicited by 8-Br-cAMP and H89 on cells transfected with either WT or AE3-A867D is very similar, suggesting that the Ala to Asp mutation does not affect the activity modulation mediated by the cytoplasmic domain but rather decreases the overall transport capacity of the protein.

How could a reduction of AE3 Cl⁻/HCO₃⁻ exchange activity underlie some cases of IGE? AE3 has a broad expression pattern in the mouse brain with a particularly strong presence in the stratum pyramidale of the hippocampal CA3 region (23). This region contains the cell bodies of the pyramidal neurons, which are the principal excitatory neurons of the hippocampus and are considered to be the “pacemaker” of this structure (49). Much of the synchronous bursting activity, associated with intervals between seizures, appears to be generated in CA3 (19, 49). AE3 Cl⁻/HCO₃⁻ exchange activity reduces cytosolic HCO₃⁻ levels with a concomitant intracellular increase in Cl⁻ concentration. If AE3 transport occurs in a confined space or area with low perfusion, a depletion of extracellular Cl⁻ and an increase of HCO₃⁻ will occur. As a consequence of these direct effects, AE3-mediated efflux of HCO₃⁻ from the cytosol will acidify the cell and alkalize the extracellular space. Decreased AE3 activity would result in altered intra- and extracellular pH as well as Cl⁻ levels. In turn this could alter the ligand-binding properties of receptors in the extracellular milieu and/or signal transduction processes in the cytosol. Our observation that the A867D mutation reduces AE3 activity is consistent with the increased sensitivity to chemically induced seizures seen in AE3⁺/⁻ mice (23).

Can changes of cytosolic or extracellular Cl⁻, HCO₃⁻ or pH cause epilepsy? Interestingly, mice null for the KCC2 neuron-specific K⁺/Cl⁻ cotransporter display neuronal hyperexcitability (50) and increased susceptibility to seizures (51). KCC2 normally functions to maintain low cytosolic Cl⁻ levels in an electroneutral manner. Since glycine and GABA receptors both conduct Cl⁻, this shifts their reversal potentials to more negative values (50). Analogously, reduced Cl⁻ accumulating AE3 activity (as in the A867D mutant) would reduce the driving force for the two Cl⁻-conducting receptors mentioned above, but in the opposite direction, since a reduced AE3 activity will decrease the amount of intracellular Cl⁻. A role for pH regulation in control of epilepsy is suggested by the spontaneous mouse mutant of the alkalizing Na⁺/H⁺ exchanger (NHE1), which displays slow wave epilepsy; this could, however be secondary to brain structural changes, rather than a result of alkalizing capacity (16). Finally, mice with a targeted gene disruption of the electroneutral Na⁺/HCO₃⁻ co-transporter SLC4A10 (36), a relative of AE3 (SLC4A3), have markedly reduced sensitivity to chemically induced seizures (24). Thus disruption of HCO₃⁻-accumulating SLC4A10 function reduces seizures, whereas loss of AE3’s HCO₃⁻ efflux function promotes them. Since SLC4A10 does not, under physiological conditions, have a Na⁺-driven Cl⁻/HCO₃⁻ exchange activity but mediates an electroneutral Na⁺/HCO₃⁻ cotransport, the neuronal [Cl⁻]i levels are likely to remain unaffected (36). Together this implies that the acidifying HCO₃⁻ efflux capacity of AE3 is the function that is needed to help suppress epileptic seizures (24, 50).

Epilepsy affects about 50 million people worldwide and nearly 30% of these patients are affected by IGE (43, 45). Epileptic seizures occur when neurons keep firing instead of transmitting electrical pulses in a controlled manner. This disturbance in brain electrical excitability can result in invol-
untary spasmodic muscle contractions and unconsciousness (43). Neuronal activity and metabolism generate important changes in intracellular and extracellular pH (13). Many ion channels are sensitive to pH, therefore, the tight modulation and control of pH is necessary for proper brain function, which is maintained by several proteins (23). Among these are the Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanger AE3 (46).

Genome-linkage analysis mapped a susceptibility locus for IGE to the chromosomal region containing the AE3 gene (39). Subsequently, a high frequency of the A867D allele variant is maintained by several proteins (23). Among these is the neuronal cultures.

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