Regulator of G Protein Signaling Protein Suppression of Gαo Protein-Mediated α2A Adrenergic Receptor Inhibition of Mouse Hippocampal CA3 Epileptiform Activity


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ABSTRACT

Activation of G protein-coupled α2 adrenergic receptors (ARs) inhibits epileptiform activity in the hippocampal CA3 region. The specific mechanism underlying this action is unclear. This study investigated which subtype(s) of α2ARs and G proteins (Gαo or Gαi) are involved in this response using recordings of mouse hippocampal CA3 epileptiform bursts. Application of epinephrine (EPI) or norepinephrine (NE) reduced the frequency of bursts in a concentration-dependent manner: (−)EPI > (−)NE >> (+)NE. To identify the α2AR subtype involved, equilibrium dissociation constants (pKd) were determined for the selective α2AR antagonists atipamezole (8.79), rauwolscine (7.75), 2-(2,6-dimethoxyphenoxoxyethyl)aminomethyl-1,4-benzodioxane hydrochloride (WB-4101; 6.87), and prazosin (5.71). Calculated pKd values correlated best with affinities determined previously for the mouse α2AR subtype (r = 0.98, slope = 1.07). Furthermore, the inhibitory effects of EPI were lost in hippocampal slices from α2A-AR- but not α2C-AR-knockout mice. Pretreatment with pertussis toxin also reduced the EPI-mediated inhibition of epileptiform bursts. Finally, using knock-in mice with point mutations that disrupt regulator of G protein signaling (RGS) binding to Gαo subunits to enhance signaling by that G protein, the EPI-mediated inhibition of bursts was significantly more potent in slices from RGS-insensitive Gαo GT184S heterozygous (Gαo+/-/GS) mice compared with either Gαo G184S heterozygous (Gαo+/-/GS) or control mice (EIC50 = 2.5 versus 19 and 23 nM, respectively). Together, these findings indicate that the inhibitory effect of EPI on hippocampal CA3 epileptiform activity uses an α2AR/Gαo protein-mediated pathway under strong inhibitory control by RGS proteins. This suggests a possible role for RGS inhibitors or selective α2AAR agonists as a novel antiepileptic drug therapy.

The noradrenergic system modulates many physiological and pathological processes within the central nervous system (CNS). Noradrenergic neurons regulate attention and arousal, sleep, and learning and memory (Pupo and Minneman, 2001) and seem to attenuate epileptic activity (Giorgi et al., 2004). The hippocampus receives substantial noradrenergic innervation in all regions, including the cornu ammonis 3 (CA3), a region essential for many cognitive functions such as spatial pattern recognition, novelty detection, and short-term memory (Kesner et al., 2004). The CA3 region possesses a dense recurrent network of excitatory axons between the pyramidal neurons that may be crucial for performing these

ABBREVIATIONS: CNS, central nervous system; ACSF, artificial cerebral spinal fluid; AR, adrenergic receptor; CA3, cornu ammonis 3; EPI, epinephrine; GPCR, G-protein coupled receptor; KO, knockout; NE, norepinephrine; RGS, regulator of G-protein signaling; WB-4101, 2-(2,6-dimethoxyphenoxoxyethyl)aminomethyl-1,4-benzodioxane hydrochloride; WT, wild type; PTX, pertussis toxin; JP-1302, N-[4-[4-methyl-1-piperazinyl]phenyl]-9-acridinamine dihydrochloride.

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cognitive functions but also makes the region vulnerable to overexcitation (Schwartzkroin, 1986). This region has one of the lowest seizure thresholds and is often involved in temporal lobe epilepsy, the most common human epileptic syndrome. It is clear that thoroughly delineating the inhibitory and excitatory aspects of this region is critical to understanding CNS function and dysfunction and to designing targeted therapeutic approaches.

Norepinephrine (NE) is the major neurotransmitter released by noradrenergic neurons and modulates several CA3 processes. NE has been shown to facilitate long-term potentiation, which is involved in memory formation, and antiepileptic activity (Giorgi et al., 2004) in the hippocampal CA3 region. Increased NE release in the brain has been shown to inhibit epileptiform activity, whereas reduced NE levels seem to increase seizure susceptibility (Weinshenker and Szot, 2002). Although the mechanism by which NE mediates these effects is still unclear, NE may both potentiate memory and inhibit the overexcitation associated with seizures (Jurgens et al., 2005) through the distinct and diverse expression of postsynaptic receptor subtypes (Hillman et al., 2005).

Adrenergic receptors (ARs) are divided into three major classes, each of which has a unique G protein pairing resulting in diverse physiological actions (Pupo and Minneman, 2001). Studies have suggested that βARs mediate the enhancement of long-term potentiation (Hopkins and Johnston, 1988) and memory (Devagues and Sara, 1991), whereas the antiepileptogenic actions of NE may involve α2AR activation (Giorgi et al., 2004). Pharmacological and molecular cloning studies have revealed the existence of three α2AR subtypes denoted α2A, α2B, and α2C (Bylund et al., 1994). We recently showed that NE inhibits rat hippocampal CA3 epileptiform bursts through α2AAR activation (Jurgens et al., 2007). Furthermore, specific activation of α2ARs attenuates seizures in mice elicited by chemoconvulsants (Szot et al., 2004).

ARs are part of a large and diverse family of GTP-binding (G) protein-coupled receptors (GPCRs). The extracellular signals received by GPCRs are relayed by heterotrimeric G proteins (Gαβγ) to effector enzymes and channels within the cell (Gilman, 1987). The conversion of GDP-bound inactive Gαβγ heterotrimer into activated Ga-GTP and Gβγ subunits is achieved by catalyzing nucleotide exchange on Ga subunits via GPCR activation. Once released, the subunits interact with a variety of downstream effectors in an intracellular signaling cascade (Offermanns, 2003). Deactivation of the G protein is achieved by hydrolysis of the Ga-bound GTP, a step that controls the duration of the signal. The GDP-bound Ga subunit will then reform with the G-βγ heterodimer, forming an inactive trimer once again.

For some Gα families (Gαi and Gαs), the rate of GTP hydrolysis can be enhanced by regulator of G protein signaling (RGS) proteins (Berman et al., 1996; Watson et al., 1996). Consequently, RGS proteins are negative modulators of signaling through receptors coupled to the Gi or Gs family of G proteins (Clark et al., 2008) and enhance intrinsic GTPase activity of the GTP-bound Ga subunits. This GTPase acceleration attenuates G protein signaling by resetting the Ga subunit to its inactive conformation (Hollinger and Hepler, 2002). Interfering with the activity of RGS proteins allows the Gs subunit to remain active for a longer time, effectively enhancing the signal (Lan et al., 1998; Clark et al., 2003). Therapeutic agents targeting RGS proteins could be used to enhance the effect of current GPCR-mediated drug therapies by reducing the required therapeutic dose while increasing the regional agonist specificity, thereby decreasing the possibility of side effects (Zhong and Neubig, 2001; Neubig and Siderovski, 2002).

This study investigated the role of α2ARs and RGS proteins in the antiepileptic actions of NE using field recordings of hippocampal CA3 epileptiform burst activity and a combination of selective blockers for the AR and G protein subtypes, transgenic α2AR knockout, and RGS-insensitive Gαi subunit knock-in mice. Delineating which α2AR and G protein subtypes are involved in attenuating hippocampal epileptiform activity will help to further elucidate the mechanism by which NE inhibits epileptogenesis and may suggest potential targets for antiepileptic drug therapy.

### Materials and Methods

#### Reagents

Atipamezole was made by Orion Corporation (Espoo, Finland). Desipramine, L-(-)-epinephrine (−)-bitartrate, L-(-)-norepinephrine (+)-bitartrate, D(+)-norepinephrine (-)-bitartrate, oxymetazoline hydrochloride, perrussit tocin, picrotxin, pindolol, and timolol maleate were obtained from Sigma-Aldrich (St. Louis, MO). Prazosin hydrochloride, rauwolscine hydrochloride, and WB-4101 were acquired from Tocris Cookson Inc. (Ellisville, MO). All chemical reagents used to make the artificial cerebrospinal fluid (ACSF) were of biological grade from J. T. Baker, Inc. (Phillipsburg, NJ) or Thermo Fisher Scientific (Waltham, MA). Isoflurane was purchased from Abbott Diagnostics (Chicago, IL).

#### Animals

C57BL/6J mice of both genders were used in the present study. Mice were housed two to four per cage (size 11.5 × 7 inches) under standard laboratory conditions on a 12-h light/dark cycle (lights on at 7:00 AM) in rooms maintained at a temperature of 22°C with a relative humidity of ~55%. Water and dried laboratory food (Teklad Global 18% Protein Rodent Diet; Harlan Teklad, Madison, WI) were provided ad libitum. Mice were allowed to acclimate for at least 4 days (arrival see below). All protocols described were approved by the Institutional Animal Care and Use Committee of Emory University (Atlanta, GA), the University of Michigan (Ann Arbor, MI), and the University of North Dakota (Grand Forks, ND) in accordance with National Institute of Health guidelines (Institute of Laboratory Animal Resources, 1996) and meet the guidelines of the American Association for Accreditation of Laboratory Animal Care.

#### Transgenic Mice

**Generation of α2A- and α2C-AR-Knockout Mice**

Mice were housed two to four per cage (size 11.5 × 7 inches) under standard laboratory conditions on a 12-h light/dark cycle (lights on at 7:00 AM) in rooms maintained at a temperature of 22°C with a relative humidity of ~55%. Water and dried laboratory food (Teklad Global 18% Protein Rodent Diet; Harlan Teklad, Madison, WI) were provided ad libitum. Mice were allowed to acclimate for at least 4 days (arrival see below). All protocols described were approved by the Institutional Animal Care and Use Committee of Emory University (Atlanta, GA), the University of Michigan (Ann Arbor, MI), and the University of North Dakota (Grand Forks, ND) in accordance with National Institute of Health guidelines (Institute of Laboratory Animal Resources, 1996) and meet the guidelines of the American Association for Accreditation of Laboratory Animal Care.

**Generation of α2A+/- and α2C+/- Mice**

Generation of α2A- and α2C-knockout mice, α2A(-/-) [α2A/α2C: (−)/(−)] and α2C(-/-) [α2A/α2C: (+)/(−)] mice, maintained on a pure C57BL/6J background, were generated at Emory University using heterozygous α2A(-/-) and α2C(-/-) mice obtained from Brian K. Kobilka (Stanford University, Stanford, CA). Genotypes were confirmed by polymerase chain reaction. All mice were reared in a specific pathogen-free facility at Emory University with a 12-h light/dark cycle (lights on at 7:00 AM) and were shipped to the University of North Dakota at age 2 to 5 months. Control animals used in these studies were wild-type (WT) C57BL/6J [α2A/α2C: (+)/(+)] mice purchased from The Jackson Laboratory (Bar Harbor, ME).

**Generation of Gα184S**

This study investigated the role of α2ARs and RGS proteins in the antiepileptic actions of NE using field recordings of hippocampal CA3 epileptiform burst activity and a combination of selective blockers for the AR and G protein subtypes, transgenic α2AR knockout, and RGS-insensitive Gαi subunit knock-in mice. Delineating which α2AR and G protein subtypes are involved in attenuating hippocampal epileptiform activity will help to further elucidate the mechanism by which NE inhibits epileptogenesis and may suggest potential targets for antiepileptic drug therapy.
et al., 2006). Specifically, we prepared a targeting construct by restriction digestion to obtain DNA fragments of the mouse Nmna gene from a BAC clone derived from 129-C57 DNA Bac library (ResGen; Invitrogen, Carlsbad, CA). Using those fragments, a targeting construct was prepared in the TKLNL vector (Mortensen et al., 1992). First the mutant Gαo exon 5 was produced by mutating the sequence AAACCAACTG GCCATCGTAA GAA to AAACCAAGTACTGTA GAAA. The bases in boldface type indicate the changed codon (Gly184 to Ser184), and the underline portion designates the location of the resulting diagnostic SpeI restriction site. This mutated exon 5 and additional 5' genomic sequence to form the "left" homology arm was cloned into TKLNL to introduce the loxP-flanked neo marker after resulting diagnostic SpeI restriction site. This mutated exon 5 and CO2. Using a conventional tissue sectioning apparatus (Stoelting, 2006). CJ7 ES cells were electroporated with the targeting vector, verification of chimeric offspring, the mice were backcrossed onto a mouse blastocysts to generate ES cell-mouse chimeras. After identification of the Gαo(G184S) targeting vector (Fu et al., 2006; Huang et al., 2006). CJ7 ES cells were electroporated with the targeting vector, and homologous recombinants were isolated. Targeted CJ7 ES cells were microinjected into C57BL/6JCr × (C57BL/6 × DBA/2J)F1 mouse blastocysts to generate ES cell-mouse chimeras. After identification of chimeric offspring, the mice were backcrossed onto a C57BL/6J background for at least four generations. Only heterozygous offspring of crosses between Gαo+/G184S) male and C57BL/6J female mice (N4-N5) were used in these studies because homozygous Gαo(G184S/G184S) offspring from heterozygous × heterozygous crosses were not viable. Control animals used in these studies were WT littermates (+/+). The Gαo(G184S) heterozygous (Gαo+/-/G184S) knock-in mice, maintained on a pure C57BL/6J background (>10 generations), were generated at the University of Michigan, Ann Arbor, as described previously (Fu et al., 2006). All genotypes were confirmed by polymerase chain reaction. Control animals used in these studies were WT C57BL/6J mice from The Jackson Laboratory. Both the Gαo(G184S) and Gαo(G184S) heterozygous (+/-) knock-in mice were reared at the University of Michigan and were confirmed to be pathogen-free before their shipping to the University of North Dakota at age 1 to 3 months.

Hippocampal Slice Preparation

After being deeply anesthetized with isoflurane, mice weighing 16 to 27 g were decapitated, and their brains were rapidly removed. The hippocampi were then quickly dissected from each hemisphere and placed into an ice-cold Ringer solution containing 110 mEq choline chloride, 2.5 mM KCl, 7 mM MgSO4, 0.5 mM CaCl2, 1.25 mM NaH2PO4, 25 mM NaHCO3, 25 mM t-glucose, 11.6 mM sodium ascorbate, and 3.1 mM sodium pyruvate, saturated with 95% O2/5% CO2. Using a conventional tissue sectioning apparatus (Stoelting, Wood Dale, IL), the hippocampi were sliced transversely into 500-μm thick sections and transferred to ACSF consisting of 119 mM NaCl, 5 mM KCl, 1.3 mM MgSO4, 2.5 mM CaCl2, 1 mEq NaH2PO4, 26.2 mM NaHCO3, and 11 mM t-glucose, which was continually aerated with 95% O2/5% CO2. The slices were incubated at 32 ± 1°C for 30 min, then transferred to room temperature (22 ± 1°C) and allowed to recover for at least 30 min before experimentation.

Electrophysiological Recordings

A single slice was transferred to the recording chamber, where it was submerged and superfused continuously at a rate of at least 4 m/min with ACSF at room temperature. Glass microelectrodes were made using a vertical two-stage puller (PP-830; Narishige, Tokyo, Japan). Extracellular field potentials were recorded using microelectrodes filled with 3 M NaCl placed in the stratum pyramidale of the CA3 region of the hippocampus using an SMI-61 stereo microscope (Olympus, Melville, NY). Potentials were detected using either an Axolamp 2B (Molecular Devices, Sunnyvale, CA) or BVC-700A (Dagan, Minneapolis, MN) microelectrode amplifier, amplified using a Brownlee 440 signal conditioner (Brownlee Precision, San Jose, CA), digitized with a Digidata 1322A analog-to-digital converter (Molecular Devices), and recorded using Axoscope 9.0 software (Molecular Devices).

Generation of Epileptiform Activity

Hippocampal CA3 pyramidal neurons are prone to spontaneously firing epileptiform bursts partly as a result of their extensive associative connections (Schwartzkroin, 1986). This activity was easily generated by superfusing the slice with ACSF containing 100 μM picrotoxin, a GABAß receptor blocker, to attenuate synaptic inhibition. If no burst discharges were seen after 30 min of superfusion, the slice was determined to be unresponsive and discarded. Once continuous spontaneous epileptiform burst discharges were evident, 30 min of baseline data were recorded before any exposure to an AR agonist. The ACSF also contained 0.5 μM desipramine to block NE transporters (i.e., potential reuptake of the catecholamines epinephrine (EPI) and NE) and 30 μM timolol to block any βAR-mediated excitatory effects (Jurgens et al., 2005), as well as any applicable αAR antagonist. Before being used, each AR antagonist was tested to ensure that it possessed no independent effects. Preliminary experiments also confirmed that each AR agonist concentration caused its maximal effect during an 8-min application (data not shown). Because the α2AR antagonist rauwolscine also has potent serotonergic 5-hydroxytryptamine1A receptor-mediated agonist activity (Newman-Tancredi et al., 1998), we substituted 3 μM pindolol (which blocks both βAR and 5-hydroxytryptamine1A receptors) for timolol (which only blocks βARs) when using this particular α2AR antagonist.

Data Analysis

Epileptiform burst discharge frequencies were visualized in real time (Fig. 1A) while being recorded for subsequent analysis. Postexperimentation analysis was completed using Mini Analysis 6.0 software (Synaptosoft, Decatur, GA). The last interval correlating to each agonist concentration was noted, the baseline frequency was subtracted, and that value was used to plot a concentration-response expressed as a percentage of maximal response. Frequency versus agonist concentration data were then entered into Prism 5.0 software (GraphPad Software Inc., San Diego, CA), and concentration-response curves were constructed using a nonlinear least-squares curve-fitting method. Each curve was fitted with a standard (slope = unity) or variable slope, and the best fit was determined using an F test with a value of p < 0.05. The calculated EC50 value was used as a measurement of agonist potency. Significance between groups was compared statistically using the Student’s t test (p < 0.05).

Schild analysis was used to determine the apparent equilibrium dissociation constants (pKb) for selective αAR antagonists (Arunlakshana and Schild, 1959). For each experiment, cumulative concentration-response curves were performed in adjacent slices from the same mouse (one dose-response curve per slice). Dose ratios of EC50 values were calculated in the presence and absence of a selective α2AR antagonist and Schild plots constructed by graphing the log of the dose ratio − 1 versus the log of the antagonist concentration. Linear regression analysis of these points was used to determine the slope and x-intercept. Schild regression slopes are given as mean ± S.E. and were considered to be nonunity if the 95% confidence interval did not include the value of 1. The pKb values of αAR antagonists causing competitive inhibition of the EPI-mediated reduction in burst frequencies were calculated from Schild regression x-intercepts. Differences in pKb values and Schild regression slopes were determined by analysis of covariance with a p < 0.05 level of probability accepted as significant. EC50 and pKb values are expressed as the mean ± S.E. for n experiments.

Results

Effects of EPI and NE on Mouse CA3 Epileptiform Activity. We first examined the effects of EPI on mouse CA3

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epileptiform burst discharges in the presence of timolol (βAR blockade) to elucidate the action of αAR activation on hippocampal activity. Picrotoxin-induced epileptiform burst discharges are shown in Fig. 1, and their frequency is reduced by application of EPI in a concentration-dependent manner. For this particular experiment, the EC_{50} value calculated from nonlinear regression analysis was 48 nM. Our previous work in rats has shown that this effect is most likely mediated by an α_{2A} AR in the CA3 region of the hippocampus (Jurgens et al., 2007). As illustrated in Fig. 2, the rank order of potency of the three AR agonists tested in this manner revealed that (-)EPI (31 ± 8.1 nM, n = 45 slices) > (−)NE (150 ± 45 nM, n = 15 slices) > (+)NE (4700 ± 3300 nM, n = 10 slices), which is consistent with our previous results (Jurgens et al., 2007) and the order expected for αARs.

**Effects of the Selective α_{2A} AR Antagonist Atipamezole and Subtype-Selective α_{2A} AR Antagonists on the EPI-Mediated Decrease in Burst Discharge Frequencies.** Functional determination of equilibrium dissociation constant (K_{i}) value for selective αAR antagonists was used to characterize the type of αAR mediating decreased burst frequency in the hippocampal CA3 region. Pretreatment of hippocampal slices with 3, 10, and 30 nM atipamezole produced 2-, 6-, and 22-fold parallel rightward shifts of the fitted EPI concentration-response curve (Fig. 3A). The pK_{i} of 8.79 (n = 5) for atipamezole (Fig. 3B) was similar to previously published binding pK_{i} values for the mouse α_{2A} ARs (Link et al., 1992; Chruscinski et al., 1992; see also Table 1). This result suggests that the response is mediated by an α_{2A} AR.

Subtype-selective antagonists were then used to determine the specific subtype of α_{2A} AR mediating burst frequency reduction in the mouse hippocampal CA3 region. Apparent pK_{b} values of subtype-selective α_{2A} AR competitive antagonists were determined using Schild regression analysis. Slices pretreated with either prazosin (α_{2B} AR-selective), rauwolscine (α_{2C} AR-selective), or WB-4101 (α_{2C} AR-selective) produced parallel rightward shifts of the fitted EPI concentration-response curve in all instances (data not shown). For each of these selective α_{2A} AR antagonists, the slope of the regression line was close to the value of unity. The logs of the equilibrium dissociation constants (pK_{b}) calculated for these α_{2A} AR antagonists were as follows: rauwolscine (7.75, n = 3), WB-4101 (6.87, n = 3), and prazosin (5.71, n = 4) (Table 1).

**α_{2A} AR Antagonist Functional pK_{b} Estimates Correlate to α_{2A} AR pK_{i} Values.** A method often used to compare equilibrium dissociation constants of many receptor antagonists is to correlate pK_{b} values with previously published pK_{i} values (Bylund, 1988). Both the correlation coefficient and slope of the correlation line should be close to unity if the calculated functional values correspond to the published binding constants for a specific receptor. Illustrated in Fig. 4 are the correlations between the pK_{b} values determined for the selective αAR antagonists used in this study and the previously published pK_{i} values of these AR antagonists for each mouse α_{2A} AR subtype (Table 1). For the mouse α_{2A} AR subtype, a very high correlation coefficient (r = 0.98) along with a slope similar to unity (slope = 1.07) were calculated for our experimental pK_{b} values compared with published binding affinity values (Fig. 4A). In contrast, for the mouse α_{2B} AR, a poor correlation coefficient (r = 0.88) and low slope

![Fig. 1. Effects of EPI on mouse hippocampal CA3 epileptiform activity. A, continuous 150-s long chart recordings of burst discharges recorded in the hippocampal CA3 region of brain slices from WT mice. Epileptiform burst discharges were elicited by including 100 μM concentration of the GABA_A receptor blocker picrotoxin in the perfusing ACSF containing 30 μM timolol and 0.5 μM desipramine. Under these conditions, bath application of EPI reduced burst frequency in a concentration-dependent manner from 10 bursts (0.067 Hz) in control Ringer solution to 7 (0.047 Hz) in 30 nM EPI, 3 (0.020 Hz) in 300 nM EPI, and 1 (0.007 Hz) in 3 μM EPI. B, frequency histogram of the number of burst discharges versus time of EPI application. Each bin represents the frequency averaged over an approximately 150-s epoch. Increasing concentrations of EPI were applied to the bath for the 8-min periods indicated. Inset, concentration-response curve derived from the frequency histogram. Data points were plotted as the percentage of maximal inhibition (decrease in epileptiform burst frequency), and the curve was constructed using a nonlinear least-squares curve-fitting method. For this experiment, the concentration-response curve was fit best by a nonvariable sigmoidal model with a calculated EC_{50} value for EPI of 48 nM.
These results suggest that the significant difference in the efficacy of (ual experiment best fit to a nonvariable sigmoidal curve. There was no age of decrease (reduction) in epileptiform burst frequency. Each individual experiment best fit to a nonvariable sigmoidal curve. There was no significant difference in the efficacies of (EPI (88 \pm 2.6\%), (NE (58 \pm 3.7\%), and (+)NE (58 \pm 7.2\%) at reducing epileptiform activity.

Fig. 2. Potency for EPI and NE inhibiting hippocampal CA3 epileptiform burst activity. Extracellular field potential recordings were used to generate concentration-response curves using increasing amounts of (EPI (■), (NE (●), and (+)NE (○) in the presence of 100 \mu M picrotoxin, 30 \mu M timolol, and 0.5 \mu M desipramine. There was a significant difference in the potencies (EC_{50} values) calculated for (EPI (31 \pm 8.1 \mu M, n = 45 slices from 18 animals), (NE (150 \pm 45 \mu M, n = 15 slices from 7 animals), and (+)NE (4700 \pm 3300 \mu M, n = 10 slices from 4 animals). Concentration-response curves for each agonist were plotted as a percentage of decrease (reduction) in epileptiform burst frequency. Each individual experiment best fit to a nonvariable sigmoidal curve. There was no significant difference in the efficacy of (EPI (88 \pm 2.6\%), (NE (67 \pm 3.7\%), and (+)NE (58 \pm 7.2\%) at reducing epileptiform activity.

Effects of Subtype-Selective \alpha_2AAR Antagonist Oxymetazoline on the EPI-Mediated Decrease in Burst Discharge Frequencies in \alpha_2CAR-KO Mice. To further evaluate a potential role for \alpha_2AARs and confirm that the response was primarily an \alpha_2AAR response, the selective \alpha_2AAR antagonist oxymetazoline was used in brain slices made from \alpha_2CAR-KO mice. \alpha_2CAR-KO mouse slices that had been pretreated with 100, 300, and 1000 \mu M oxymetazoline produced 6-, 22-, and 70-fold parallel rightward shifts of the fitted EPI concentration-response curve (Fig. 6A). The Schild regression slope was 1.16 (± 0.12) and the x-intercept correlating to a pK_i value of 7.50 (n = 7 animals) (Fig. 6B). The mouse \alpha_2AAR reported a pK_i value of 7.49 matched closely to our pK_i value, whereas the \alpha_2BAR and \alpha_2CAR reported pK_i values with previously published pK_i values (Fig. 4B). Likewise for the mouse \alpha_2BAR, a poor correlation coefficient (r = 0.89) and low slope (slope = 0.63) were seen (Fig. 4C). These results suggest that the \alpha_2BAR is the predominant subtype mediating the antiepileptic action of EPI in mouse hippocampus.

Effects of Subtype-Selective \alpha_2AAR Antagonist Oxymetazoline on the EPI-Mediated Decrease in Burst Discharge Frequencies in \alpha_2CAR-KO Mice.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>( pK_i )</th>
<th>Slope</th>
<th>( \alpha_2AAR )</th>
<th>( \alpha_2BAR )</th>
<th>( \alpha_2CAR )</th>
</tr>
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<tbody>
<tr>
<td>Atipamezole</td>
<td>8.79</td>
<td>1.06 ± 0.12</td>
<td>9.07^a</td>
<td>8.30^a</td>
<td>8.80^a</td>
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<tr>
<td>Rauwolscine</td>
<td>7.75</td>
<td>1.01 ± 0.07</td>
<td>7.27^a</td>
<td>7.15^b</td>
<td>9.10^c</td>
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<tr>
<td>WB-4101</td>
<td>6.87</td>
<td>0.89 ± 0.08</td>
<td>6.58^b</td>
<td>7.15^b</td>
<td>8.11^a</td>
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<tr>
<td>Prazosin</td>
<td>5.71</td>
<td>0.97 ± 0.06</td>
<td>6.67^c</td>
<td>7.23^b</td>
<td>7.01^a</td>
</tr>
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a Link et al. (1992).
b Chrusciński et al. (1992).

Fig. 3. Schild regression analysis using the selective \alpha_2AAR antagonist atipamezole. A, consecutive EPI concentration-response curves demonstrate a concentration-dependent effect of the selective \alpha_2AAR antagonist, atipamezole, on the EPI-mediated inhibition of hippocampal CA3 epileptiform activity in brain slices from WT mice. Pretreatment with 3 \mu M (■), 10 \mu M (●), and 30 \mu M (○) concentrations of this antagonist produced consecutive parallel rightward shifts of the EPI curve that were significantly different from control (EC_{50} = 76 ± 3, 218 ± 30, and 762 ± 274, respectively, versus 34 ± 12 \mu M for control). B, using dose ratios calculated from individual experiments illustrated in A, a Schild plot was created generating a regression slope equaling 1.06 ± 0.12 and an x-intercept correlating to a pK_i value of 8.79, n = 5 animals (see Table 1).
values of 5.92 (Chruscincki et al., 1992) and 6.96 (Link et al., 1992) did not. If the \( \alpha_{2B}/\alpha_{2A} \) made a significant contribution, the slope of the Schild plot should have been less than 1. These results further confirm that this response is primarily mediated by an \( \alpha_{2A}/\alpha_{2A} \).

**Effects of Pertussis Toxin on EPI-Mediated Inhibition of CA3 Epileptiform Activity.** Pertussis toxin (PTX) blocks the receptor-mediated activation of \( \alpha_{i2}/\alpha_{i2} \) proteins. We used PTX to assess which \( G \) protein type is involved in the inhibitory effects of EPI. Extracellular field potential recordings of epileptiform burst frequency were used to generate concentration-response curves using increasing amounts of EPI in untreated control slices or in slices treated with 5 \( \mu \)g/ml PTX for 7 to 8 h. As illustrated in Fig. 7, the mean concentration-response curve for nontreated control slices was fit best by a unity-slope sigmoidal model with a calculated EC\(_{50} \) value of 12 ± 3.9 nM and a maximum effect of 74 ± 6.1\% (\( n = 13 \) slices). Conversely, for PTX-treated slices from the same mice, the mean concentration-response curve showed minimal inhibition (<25\%) (\( n = 12 \) slices). These results indicate that inhibition of mouse hippocampal CA3 epileptiform activity in response to EPI is mediated by either \( G_{i1} \) or \( G_{i2} \) proteins and not \( G_{o} \) or \( G_{i2} \) proteins.

**EPI-Mediated Inhibition of CA3 Epileptiform Activity in Slices from \( \alpha_{2A}/\alpha_{2A} \) Heterozygous (\( \alpha_{i2}/\alpha_{i2} \)) and \( \alpha_{2A}/\alpha_{2A} \) Heterozygous (\( \alpha_{i2}/\alpha_{i2} \)) Knock-in Mice.** To determine a potential role of RGS proteins in the regulation of this response and which type of inhibitory G protein may be involved, we used mice with a knock-in \( G_{i2} \) subunit mutation (\( G_{i2} \)) that renders \( G_{i2} \) insensitive to RGS proteins incapable of binding to the RGS protein. This results in the loss of RGS-mediated inhibition of the \( G_{i2} \) and \( G_{i2} \) protein and enhances \( G_{i2} \)-specific effects in tissues with responses under RGS control. An increase in response with one of these RGS-insensitive \( G \) proteins would indicate that \( G \) protein as contributing to the response and RGS proteins as negative regulators. As before, WT control, \( \alpha_{i2}/\alpha_{i2} \), or \( \alpha_{i2}/\alpha_{i2} \) slices were pretreated with the \( \alpha \)AR blocker timolol, and NE transporter reuptake inhibitor desipramine. Extracellular field potential recordings were used to generate concentration-response curves using increasing amounts of EPI. Inhibition of frequency burst discharges was significantly more potent in brain slices from \( \alpha_{i2} \) mice, with an EC\(_{50} \) of 2.5 ± 0.9 nM (\( n = 23 \) slices) versus litter mate control mice (EC\(_{50} \) = 19 ± 5 nM, \( n = 21 \) slices) (Fig. 8A). In contrast, there was no significant difference in \( \alpha_{2A}/\alpha_{2A} \) mice (EC\(_{50} \) = 19 ± 5 nM, \( n = 32 \) slices) compared with the WT controls (EC\(_{50} \) = 23 ± 7 nM, \( n = 22 \) slices) (Fig. 8B). These results indicate that the EPI-mediated inhibition of mouse hippocampal CA3 epileptiform activity involves a \( \alpha_{2A}/\alpha_{2A} \) mechanism under strong negative regulation by RGS proteins.

**Discussion**

The role of catecholamines in seizures and epilepsy is complicated, but it is clear that endogenous EPI and NE can protect against many types of seizures (Weinshenker and Szot, 2002). Agonists at all three types of AR (\( \beta \), \( \alpha_1 \), and \( \alpha_2 \)) can be antiepileptic, but the most consistent findings show that \( \alpha_2 \)AR agonists are generally anticonvulsant, and selective \( \alpha_2 \)AR antagonists are proconvulsant (Weinshenker and Szot, 2002). Consequently, we focused the current study on the hippocampus, which plays an important role in the common clinical condition of temporal lobe seizures, to begin to dissect mechanisms underlying the antiepileptic actions of \( \alpha_2 \)AR agonists. We used both pharmacological and mouse genetic models to define the receptor and G protein involved in the EPI-mediated antiepileptiform activity in the hippocampus. We have confirmed the role of the \( \alpha_{2A}/\alpha_{2A} \) in inhibition of hippocampal CA3 epileptiform activity in mice, as shown previously by a pharmacological approach in rats (Jurgens et al., 2007). We built upon these findings by demonstrating that this involves a PTX-sensitive \( \alpha_{2A}/\alpha_{2A} \) G protein. Furthermore, using RGS-insensitive \( \alpha \)AR subunit mutant knock-in mice, we show that endogenous RGS protein action on \( \alpha_{2A} \) strongly suppresses this signal, implicating \( \alpha_{2A} \) as a mediator of the response. In contrast, \( \alpha_{2A} \) seems not to be involved. These findings enhance our understanding of the mechanism underlying \( \alpha_{2A}/\alpha_{2A} \)-mediated inhibition of hippocampal epileptiform activity by NE and suggest a novel approach to antiepileptic drug therapies.

The \( \alpha_{2A} \)-AR is the predominant \( \alpha_{2A} \)-AR in the CNS, and it has been implicated as the primary anticonvulsant \( \alpha_{2A} \)-AR in rat hippocampus in vitro (Jurgens et al., 2007) and in mouse in vivo (Janumpalli et al., 1998). A previous study using dopamine \( \beta \)-hydroxylase, \( \alpha_{2A} \)-AR, and \( \alpha_{2C} \)-AR-KO mice showed that the proconvulsant effects of \( \alpha_{2A} \)AR agonists were mediated by the \( \alpha_{2A} \)-AR autoreceptor, which decreases NE release, whereas the anticonvulsant effects of \( \alpha_{2A} \)-AR agonists were
mediated by \( \alpha_{2A} \) ARs on target neurons (Szot et al., 2004). In the present study, we confirm the results of these findings in mouse using both pharmacological (agonist \( pK_a \)) and genetic (\( \alpha_{2A} \)-AR- and \( \alpha_{2C} \)-AR-KO) approaches. Despite expression of the \( \alpha_{2C} \)-AR in hippocampus, it does not seem to contribute at all to the antiepileptiform activity of EPI and NE (Fig. 5). Likewise, the \( \alpha_{2B} \)-AR does not seem to play a role (Fig. 6). Neither were \( \alpha_{2A} \)-ARs involved in this particular response (Fig. 3 and Table 1). This level of receptor subtype-specificity does not, however, provide any significant therapeutic advance on its own, because the \( \alpha_{2A} \)-AR is also the major receptor involved in the antihypertensive therapeutic effect of \( \alpha_{2A} \)-AR agonists and in their major sedative side effect as well (MacMillan et al., 1998). Thus, we pursued subsequent steps in the downstream signaling.

The \( \alpha_{2A} \)-ARs are known to couple primarily to \( G_{i/o} \) family G proteins with subsequent actions on several effector systems, including inhibition of adenylyl cyclase, inhibition of voltage-gated calcium channels, and activation of G protein-coupled inwardly rectifying K+ currents (Offermanns, 2003). The \( G_{i/o} \) protein family is also strongly regulated by the 20-plus member RGS protein family (Neubig and Siderovski, 2002), which has been implicated as a potential drug target (Zhong and Neubig, 2001; Roman et al., 2007). We first confirmed that the \( \alpha_{2A} \)-AR response in hippocampus was PTX-sensitive, indicating a role for the \( G_{i/o} \) family. The small residual effect after PTX treatment (<1/3 of the control response) could be due to incomplete modification of the \( G_{i/o} \) proteins during the 7- to 8-h pretreatment period, because many studies use an overnight (>12 h) treatment with PTX. Alternatively, a non-PTX-sensitive protein like \( G_z \) could play a small role.

To further examine which \( G_{i/o} \)-subtypes can mediate EPI’s effect, mice with knock-in mutant RGS-insensitive \( G_{o} \) or \( G_{i2} \) were used. The knock-in mice differ from WT only in the presence of the G184S mutation, which prevents RGS binding to the \( G_{o} \) subunit and the subsequent GTPase acceleration (Fu et al., 2004; Huang et al., 2006). Consequently, this mutation results in prolonged and enhanced activation of the modified G protein, which increases signal transduction by both the \( \alpha \) and \( \beta_{7} \) subunits derived from that G protein. The heterozygous \( G_{o} \)-, \( G_{i2} \)-, or \( G_{o2} \)-RGS-insensitive [\( G_{o} \)K(+G184S)] knock-in animals showed a 7-fold leftward shift of the EPI dose-response curve (2.5 versus 19 nM), whereas there was no significant difference in potency between the heterozygous \( G_{o2} \)-RGS-insensitive mouse (19 nM) and its control (23 nM). The pronounced effect even in the heterozygous mouse is not surprising. RGS proteins can accelerate G protein deactivation nearly 1000-fold (Mukhopadhyay and Ross, 1999; Lan et al., 2000), dramatically suppressing G protein signaling. The G184S mutation eliminates this negative regulatory effect, so it produces a gain-of-function phenotype in these mice.

**Fig. 5.** Effects of EPI on hippocampal CA3 epileptiform activity in brain slices from \( \alpha_{2A} \)-AR- and \( \alpha_{2C} \)-AR-KO mice. Extracellular field potential recordings of epileptiform burst frequency were used to generate concentration-response curves using increasing amounts of EPI in the presence of 100 \( \mu M \) picrotoxin, 30 \( \mu M \) timolol, and 0.5 \( \mu M \) desipramine. Concentration-response curves for EPI were plotted as a percentage of decrease (reduction) in epileptiform burst frequency. For the \( \alpha_{2C} \)-AR-KO mice, the mean concentration-response curve for 41 slices from 12 animals was fit best by a nonvariable sigmoidal model with a calculated EC50 value of 37 ± 12 nM, which was not significantly different from the potency of 31 ± 8.1 nM calculated for EPI in slices from WT mice (see Fig. 2). The efficacy of EPI at reducing the frequency of epileptiform bursts in slices from \( \alpha_{2C} \)-AR-KO mice was 64 ± 3.9%, which was significantly different from the 8.7 ± 3.3% inhibition for EPI in slices from \( \alpha_{2A} \)-AR-KO mice.

**Fig. 6.** Schild regression analysis using the selective \( \alpha_{2A} \)-AR ligand oxymetazoline in slices from \( \alpha_{2A} \)-AR-KO mice. A, consecutive EPI concentration-response curves demonstrate a concentration-dependent effect of the selective \( \alpha_{2A} \)-AR ligand, oxymetazoline, on the EPI-mediated inhibition of hippocampal CA3 epileptiform activity in brain slices from \( \alpha_{2A} \)-AR-KO mice. Pretreatment with 100 nM (●), 300 nM (□), and 1000 nM (○) concentrations of this antagonist produced consecutive parallel rightward shifts of the EPI curve that were significantly different from control (□) (EC50 = 205 ± 48, 738 ± 267, and 2317 ± 980 nM, respectively, versus 33 ± 9 nM for control). B, using dose ratios calculated from individual experiments illustrated in A, a Schild plot was generated, yielding a regression slope equaling 1.16 ± 0.12 and an x-intercept correlating to a \( pK_i \) of 7.50, \( n = 7 \) animals. This \( pK_i \) value matched the binding affinity of oxymetazoline (\( pK_i = 7.49 \)) for the mouse \( \alpha_{2A} \)-AR, but not the mouse \( \alpha_{2B} \)-AR (\( pK_i = 6.96 \)) (Link et al., 1992).
which even half of the G protein removed from this suppression could produce a marked increase in signaling. Previous studies with the Gα12<sub>G184S</sub> knock-in mutants have also shown significant effects in heterozygous mice (Huang et al., 2006). Thus, these results show that RGS proteins play a key role in regulating the α2AAR-mediated hippocampal CA3 antiepileptiform effect and suggest that the Gα<sub>i2</sub> subtype of G<sub>αi</sub> proteins is involved in the signaling mechanism, whereas Gα<sub>o</sub> seems not to be. At this stage, we cannot rule out a contribution from other pertussis toxin-sensitive G proteins such as Gα<sub>13</sub> or Gα<sub>23</sub>, but the evidence clearly indicates that Gα<sub>o</sub> does play a role.

Several important questions remain. Although the Gα<sub>o</sub> RGS-insensitive mouse shows that an RGS protein is involved in this system, it does not reveal which of the 20-plus RGS proteins (Hollinger and Hepler, 2002; Neubig and Siderovski, 2002) are important. Given that nearly 15 different RGS proteins can function as a GTPase-activating protein for Gα<sub>o</sub>, it may be difficult to establish which one(s) are involved. Furthermore, it is possible that more than one RGS protein may work in a redundant manner in this system. That said, the RGS7 family of RGS proteins (RGS6, 7, 9, and 11) represent intriguing candidates because they are relatively selective for Gα<sub>o</sub> in vitro (Lan et al., 2000). A second question is whether the same enhancement of α2AAR agonist anticonvulsant actions will be seen in vivo. Studies are currently underway to assess this question.

The present study suggests two strategies that may provide improved therapeutics for adrenergic agonist anticonvulsants. First, an α2AAR agonist that can selectively activate Gα<sub>i2</sub> versus Gα<sub>o</sub> or other G<sub>i</sub> family members could lead to improved potency and/or reduced side effects. It would also be important for such a compound to preferentially activate the α2AARs on target neurons over α2AAR autoreceptors that would decrease NE release. This could be achieved by a “functionally selective” (Urban et al., 2007) α2AAR agonist. Second, RGS proteins have been implicated as potential therapeutic targets. Several peptide (Jin et al., 2004; Young et al., 2004; Roof et al., 2006) and nonpeptide (Roman et al., 2007) RGS inhibitors have been described. To date, none are active in vivo for pharmacological studies, but the identification of the involved RGS protein and the creation of an inhibitor that could target it could either produce anticonvulsant effects through endogenous NE or potentially reduce side ef-

![Fig. 7. PTX reduces the EPI-mediated inhibition of hippocampal CA3 epileptiform bursts. Extracellular field potential recordings of epileptiform burst frequency were used to generate concentration-response curves using increasing amounts of EPI in untreated control slices (●) or slices treated (○) with 5 μg/ml PTX for 7 to 8 h. Concentration-response curves for EPI were plotted as a percentage of decrease (reduction) in epileptiform burst frequency. The mean concentration-response curve for nontreated control slices was fit best by a nonvariable sigmoidal model with a calculated EC<sub>50</sub> value of 12 ± 3.9 nM and an efficacy of 74 ± 6.1% (n = 13 slices from 6 animals). In contrast, for PTX-treated slices from these same mice, the mean concentration-response curve was fit best by a linear regression line and had an efficacy of 24 ± 13% (n = 12 slices from 6 animals).]

![Fig. 8. EPI-mediated inhibition of hippocampal CA3 epileptiform bursts is significantly enhanced in brain slices from Gα<sub>o</sub>/GS mice but not Gα<sub>i2</sub>/GS mice. Extracellular field potential recordings were used to generate concentration-response curves using increasing amounts of EPI (●) in the presence of 30 μM timolol and 0.5 μM desipramine. Concentration-response curves for EPI were plotted as a percent reduction in epileptiform burst frequency. Each individual experiment best fit to a nonvariable sigmoidal curve. A, there was a significant difference in the potencies (EC<sub>50</sub> values) calculated for EPI in brain slices from Gα<sub>o</sub>/GS mice (●) (2.5 ± 0.9 nM, n = 23 slices from 6 animals) versus litter mate control mice (●) (19 ± 5 nM, n = 21 slices from 6 animals). B, in contrast, the EPI-mediated inhibition of epileptiform activity was unchanged in brain slices from Gα<sub>i2</sub>/GS mice (●) (19 ± 5 nM, n = 32 slices from 6 animals) compared with WT control mice (●) (23 ± 7 nM, n = 22 slices from 8 animals). There was no significant difference in the efficacy of EPI among these four groups (Gα<sub>o</sub>/GS, 74 ± 3.8%; Gα<sub>i2</sub> litter mate control, 74 ± 4.4%; Gα<sub>i2</sub>/GS, 73 ± 2.8%; Gα<sub>o</sub>/WT control, 75 ± 3.6%).]
fects on the treatment with α2AAR-selective agonists in patients with epilepsy.

In summary, we have defined the receptor (α2AAR), a G protein (Grα1), and a regulatory mechanism (RGS proteins) that are important for the antiepileptiform actions of NE and EPI in the hippocampus, a key site of seizure activity in many patients. These advances provide a theoretical rationale for future, novel therapeutic approaches.

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