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 (pKb) were determined for the selective α2AR antagonists atipamezole (8.79), rauwolscine (7.75), 2-(6-dimethoxyphenoxoethyl)aminomethyl-1,4-benzodioxane hydrochloride (WB-4101; 6.87), and prazosin (5.71). Calculated pKb 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 α2AR-knockout mice but not α2CAR-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(G184S) heterozygous (Gαo+/+;GS) mice compared with either Gαo(G184S) heterozygous (Gαo+/+;GS) or control mice (EC50 = 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 α2AR agonists as a novel antiepileptic drug therapy.

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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-(6-dimethoxyphenoxoethyl)aminomethyl-1,4-benzodioxane hydrochloride; WT, wild type; PTX, pertussis toxin; JP-1302, N-[4-[4-methyl-1-piperazinyl]phenyl]-9-acridinamine dihydrochloride.
Consequently, RGS proteins are negative modulators of G protein signaling (RGS) proteins (Berman et al., 1996; Watson et al., 1996). Lysosome can be enhanced by regulator of G protein signaling (H9251 denoted an inactive trimer once again. Enhancing the signal (Lan et al., 1998; Clark et al., 2003). Furthermore, specific activation of α2AAR activation showed that NE inhibits rat hippocampal CA3 epileptiform bursts through α2AAR activation (Jurgens et al., 2007). Moreover, specific activation of α2AARs attenuates seizures in mice elicited by chemical convulsants (Szt, et al., 2004).

ARs are part of a large and diverse family of 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 Gaβγ heterotrimer into activated Ga-GTP and G-βγ subunits is catalyzed by catalytic nucleotide exchange on Ga subunits via GPCR activation. Once released, the subunits interact with a variety of downstream effectors in an intracellular signaling cascade (Oppermanns, 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 Gαq), 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 receptors coupled to Gαi and Gαq 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 Ga 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 region 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 Ga 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, pertussis toxin, picrotoxin, 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 after 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 α2AAR- and α2CAR-Knockout Mice. α2AAR(--/--) [α2a/α2c; --/--] and α2C(--/--) [α2a/α2c; +/-/+-] mice, maintained on a pure C57BL/6J background, were generated at Emory University using heterozygous α2C(+/-) and α2AAR(+/-) 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 Goαi21848 Heterozygous (Goαi+/GS) Knock-In Mice. The original Goα (Goαi21848) ES cell line, described in Fu et al. (2004, 2006), was developed in a 129 ES cell background, which never went germline. Consequently, the Goαi21848 mouse strain was constructed from a 129-C57 ES line using methods similar to those previously reported for the Goα subline (Fu et al., 2006; Huang...
et al., 2006). Specifically, we prepared a targeting construct by restriction digestion to obtain DNA fragments of the mouse Gnao gene from a Bac clone derived from 129-CJ7 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 AAAACAACTGGCATCAGTAA to AAAACAACTAGTATCAGTAA. 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 exon 5, then the right arm genomic fragment from exons 6 to 8 was cloned 3’ of the loxP cassette in a manner similar to that for preparing 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/6Ncr1 × (C57BL/6J × 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 (+/−+/−) of the Gαo(G184S) heterozygous (+/G184S) mice.

**Generation of Gαo(G184S) Heterozygous (Gαo(+/+G184S)) Knock-In Mice.** 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 (+/G184S) 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 mM choline chloride, 2.5 mM KCl, 7 mM MgSO4, 0.5 mM CaCl2, 1.25 mM NaH2PO4, 25 mM NaHCO3, 25 mM d-glucose, 1.16 mM sodium ascorbate, and 3.1 mM sodium pyruvate, saturated with 95% O2/5% CO2. Using a conventional tissue sectioning apparatus (Stoelting, and homologous recombinants were isolated. Targeted CJ7 ES cells were microinjected into C57BL/6Ncr1 × (C57BL/6J × 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 (+/−+/−) of the Gαo(G184S) heterozygous (+/G184S) mice.

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**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 μl/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 EZ-61 stereo microscope (Olympus, Melville, NY). Potentials were detected using either an Axon Instruments (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 GABA_A 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 agonist. 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 α1AR 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 α1AR antagonist.

**Data Analysis.**

Epileptiform burst discharge frequencies were visualized in real time (Fig. 1A) while being recorded for subsequent analysis. Postexperimen- tal analysis was completed using Mini Analysis 6.0 software (Synaptosoft, Decatur, GA). The last interval corresponding to each agonist concentration was noted, the baseline frequency was subtracted, and that value was used to plot a concentration-response curve. 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 α1AR 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 α1AR 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 α1AR 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
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_2AR 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_2AR Antagonist Atipamezole and Subtype-Selective α_2AR 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_2ARs (Link et al., 1992; Chruscinski et al., 1992; see also Table 1). This result suggests that the response is mediated by an α_2AR.

Subtype-selective antagonists were then used to determine the specific subtype of α_2AR mediating burst frequency reduction in the mouse hippocampal CA3 region. Apparent pK_i values of subtype-selective α_2AR competitive antagonists were determined using Schild regression analysis. Slices pre-treated with either prazosin (α_2B/2AR-selective), rauwolscine (α_2C/2AR-selective), or WB-4101 (α_2D/2AR-selective) produced parallel rightward shifts of the fitted EPI concentration-response curve in all instances (data not shown). For each of these selective α_2AR antagonists, the slope of the regression line was close to the value of unity. The logs of the equilibrium dissociation constants (pK_i) calculated for these α_2AR antagonists were as follows: rauwolscine (7.75, n = 3), WB-4101 (6.87, n = 3), and prazosin (5.71, n = 4) (Table 1).

**α_2AR Antagonist Functional pK_i Estimates Correlate to α_2AR pK_i Values.** A method often used to compare equilibrium dissociation constants of many receptor antagonists is to correlate pK_i 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_i 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_2AR subtype (Table 1). For the mouse α_2AR 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_i values compared with published binding affinity values (Fig. 4A). In contrast, for the mouse α_2B/2AR, 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 dual experiment best fit to a nonvariable sigmoidal curve. There was no concentration-response curves for each agonist were plotted as a percent-

rate concentration-response curves using increasing amounts of (−)EPI (■), (−)NE (○), and (+)NE (□) in the presence of 100 μM picrotoxin, 30 μM timolol, and 0.5 μM desipramine. There was a significant difference in the potencies (EC50 values) calculated for (−)EPI (31 ± 8.1 nM, n = 45 slices from 18 animals), (−)NE (150 ± 45 nM, n = 15 slices from 7 animals), and (+)NE (4700 ± 3300 nM, 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 (68 ± 2.6%), (−)NE (67 ± 3.7%), and (+)NE (58 ± 7.2%) at reducing epileptiform activity.

Effects of EPI on Epileptiform Activity in Slices from α2AAR- and α2CAR-KO Knockout Mice. To confirm our pharmacological results, we examined the effects of EPI on hippocampal CA3 epileptiform activity in α2AAR- and α2CAR-KO knockout (KO) mice. As illustrated in Fig. 5, EPI was applied in increasing concentrations to hippocampal brain slices prepared from either α2AAR- or α2CAR-KO mice. The potency of EPI in the α2CAR-KO mouse line (37 ± 12 nM, n = 15) fit best with a unity-slope sigmoidal model and was not significantly different from the WT mice (see also Fig. 2). In contrast, the effects of EPI were largely abolished in brain slices from α2AAR-KO mice with a maximum effect of less than 10% inhibition. These results demonstrate that the α2AR is the predominant receptor subtype mediating the inhibitory effects of EPI in the mouse hippocampus.

Effects of Subtype-Selective α2AAR Antagonist Oxymetazoline on the EPI-Mediated Decrease in Burst Discharge Frequencies in α2CAR-KO Mice. To further evaluate a potential role for α2AARs and confirm that the response was primarily an α2AR response, the selective α2AAR antagonist oxymetazoline was used in brain slices made from α2CAR-KO mice. α2CAR-KO mouse slices that had been pretreated with 100, 300, and 1000 nM 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 pKb value of 7.50 (n = 7 animals) (Fig. 6B). The mouse α2AAR reported a pKb value of 7.49 matched closely to our pKb value, whereas the α2bAR and α2CAR reported pKb values.

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 μM picrotoxin, 30 μM timolol, and 0.5 μM desipramine. There was a significant difference in the potencies (EC50 values) calculated for (−)EPI (31 ± 8.1 nM, n = 45 slices from 18 animals), (−)NE (150 ± 45 nM, n = 15 slices from 7 animals), and (+)NE (4700 ± 3300 nM, 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 (68 ± 2.6%), (−)NE (67 ± 3.7%), and (+)NE (58 ± 7.2%) at reducing epileptiform activity.

Table 1

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<th>α2CAR</th>
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* Link et al. (1992).
* Chruscinski et al. (1992).
values of 5.92 (Chruscinski et al., 1992) and 6.96 (Link et al., 1992) did not. If the α2bAR 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 α2aAR.

**Effects of Pertussis Toxin on EPI-Mediated Inhibition of CA3 Epileptiform Activity.** Pertussis toxin (PTX) blocks the receptor-mediated activation of Gi/o proteins. We used PTX to assess which G protein types are 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 μg/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 Gi or Go proteins and not Gq or Gi proteins.

**EPI-Mediated Inhibition of CA3 Epileptiform Activity in Slices from G_o,G184S Heterozygous (G_o,+/GS) and G_o,GS Heterozygous (G_o,GS+/-) 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 Go subunit mutation (G184S) that renders Go,G184S proteins incapable of binding to the RGS protein. This results in the loss of RGS-mediated inhibition of the Go,G184S protein and enhances Go-specific effects in tissues with responses under RGS control. An increase in response with one of these RGS-insensitive G proteins would implicate that G protein as contributing to the response and RGS proteins as negative regulators. As before, WT control, Go,G184S, or Go,G184S+/- slices were pretreated with the GABA blocker picrotoxin, βAR 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 Go,G184S 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 Go,G184S 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 EPI-mediated inhibition of hippocampal CA3 epileptiform activity involves a Go 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 Sztot, 2002). Agonists at all three types of AR (β, α1, and α2) can be antiepileptic, but the most consistent findings show that α2AR agonists are generally anticonvulsant, and selective α2AR antagonists are proconvulsant (Weinshenker and Sztot, 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 α2AR 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 α2aAR 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 G_{i2}-type G protein. Furthermore, using RGS-insensitive Go subunit mutant knock-in mice, we show that endogenous RGS protein action on G_{o} strongly suppresses this signal, implicating G_{o} as a mediator of the response. In contrast, G_{o} seems not to be involved. These findings enhance our understanding of the mechanism underlying α2aAR-mediated inhibition of hippocampal epileptiform activity by NE and suggest a novel approach to antiepileptic drug therapies.

The α2aAR is the predominant α2AR in the CNS, and it has been implicated as the primary anticonvulsant α2AR in rat hippocampus in vitro (Jurgens et al., 2007) and in mouse in vivo (Janumpalli et al., 1998). A previous study using dopamine β-hydroxylase, α2bAR, and α2cAR-KO mice showed that the proconvulsant effects of α2AR agonists were mediated by the α2aAR autoreceptor, which decreases NE release, whereas the anticonvulsant effects of α2AR agonists were...
mediated by \( \alpha_2 \)ARs on target neurons (Szot et al., 2004). In the present study, we confirm the results of these findings in mouse using both pharmacological (antagonist \( pK_a \)) and genetic (\( \alpha_2 \)AR- and \( \alpha_2 \)AR-KO) approaches. Despite expression of the \( \alpha_2 \)AR in hippocampus, it does not seem to contribute at all to the antiepileptiform activity of EP1 and NE (Fig. 5). Likewise, the \( \alpha_2 \)AR does not seem to play a role (Fig. 6). Neither were \( \alpha_2 \)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 \( \alpha_2 \)AR is also the major receptor involved in the antihypertensive therapeutic effect of \( \alpha_2 \)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 \( \alpha_2 \)ARs are known to couple primarily to \( G_{\alpha_i} \) family G proteins with subsequent actions on several effector systems, including inhibition of adenyl cyclase, inhibition of voltage-gated calcium channels, and activation of G protein-coupled inwardly rectifying K+ currents (Offermanns, 2003). The \( G_{\alpha_i} \) 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 \( \alpha_2 \)AR response in hippocampus was PTX-sensitive, indicating a role for the \( G_{\alpha_i} \) family. The small residual effect after PTX treatment (<1/3 of the control response) could be due to incomplete modification of the \( G_{\alpha_i} \) 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 \( G_x \) could play a small role.

To further examine which \( G_{\alpha_i} \) subtype(s) can mediate EP1's effect, mice with knock-in mutant RGS-insensitive \( G_{\alpha_i} \) or \( G_{\beta \gamma} \) were used. The knock-in mice differ from WT only in the presence of the \( G_{184S} \) mutation, which prevents RGS binding to the \( G_{\alpha} \) 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 \( \beta \gamma \) subunits derived from that G protein. The heterozygous \( G_{184S} \), RGS-insensitive \( [G_{184S}] \) knock-in animals showed a 7-fold leftward shift of the EP1 dose-response curve (2.5 versus 19 nM), whereas there was no significant difference in potency between the heterozygous \( G_{184S} \) RGS-insensitive mouse (19 nM) and its control (23 nM). The pronounced effect even in the heterozygous mouse is not surprising. G proteins can accelerate G protein deactivation nearly 1000-fold (Mukhopadhyay and Ross, 1999; Lan et al., 2000), dramatically suppressing G protein signaling. The \( G_{184S} \) mutation eliminates this negative regulatory effect, so it produces a gain-of-function phenotype in

**Fig. 5.** Effects of EP1 on hippocampal CA3 epileptiform activity in brain slices from \( \alpha_2 \)AR- and \( \alpha_2 \)AR-KO mice. Extracellular field potential recordings of epileptiform burst frequency were used to generate concentration-response curves using increasing amounts of EP1 in the presence of 100 \( \mu M \) picrotoxin, 30 \( \mu M \) timolol, and 0.5 \( \mu M \) desipramine. Concentration-response curves for EP1 were plotted as a percentage of decrease (reduction) in epileptiform burst frequency. For the \( \alpha_2 \)AR-KO mice, the mean concentration-response curve for 41 slices from 12 animals was fit best by a linear regression line. In contrast, the mean concentration-response curve for 39 brain slices from 15 \( \alpha_2 \)AR-KO mice was fit best by a nonvariable sigmoidal model with a calculated EC\textsubscript{50} value of 37 ± 12 nM, which was not significantly different from the potency of 31 ± 8 nM calculated for EP1 in slices from WT mice (see Fig. 2). The efficacy of EP1 at reducing the frequency of epileptiform bursts in slices from \( \alpha_2 \)AR-KO mice was 64 ± 3.9%, which was significantly different from the 8.7 ± 3.3% inhibition for EP1 in slices from \( \alpha_2 \)AR-KO mice.

**Fig. 6.** Schild regression analysis using the selective \( \alpha_2 \)AR ligand oxymetazoline in slices from \( \alpha_2 \)AR-KO mice. A, consecutive EP1 concentration-response curves demonstrate a concentration-dependent effect of the selective \( \alpha_2 \)AR ligand, oxymetazoline, on the EP1-mediated inhibition of hippocampal CA3 epileptiform activity in brain slices from \( \alpha_2 \)AR-KO mice. Pretreatment with 100 nM (○), 300 nM (■), and 1000 nM (□) concentrations of this antagonist produced consecutive parallel rightward shifts of the EP1 curve that were significantly different from control (○) (EC\textsubscript{50} = 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 created generating a regression slope equaling 1.16 ± 0.12 and an x-intercept correlating to a \( pK_a \) value of 7.50, \( n = 7 \) animals. This \( pK_a \) value matched the binding affinity of oxymetazoline \((pK_a = 7.49)\) for the mouse \( \alpha_2 \)AR, but not the mouse \( \alpha_2 \)AR \((pK_a = 5.92)\) (Chruscinski et al., 1992) or mouse \( \alpha_2 \)AR \((pK_a = 6.96)\) (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 $G_{o2}^{Gi84}$ 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 $\alpha_{2A}$-mediated hippocampal CA3 antiepileptiform effect and suggest that the $G_{o}$ subtype of $G_{o}$ proteins is involved in the signaling mechanism, whereas $G_{o2}$ seems not to be. At this stage, we cannot rule out a contribution from other pertussis toxin-sensitive G proteins such as $G_{o1}$ or $G_{o3}$, but the evidence clearly indicates that $G_{o}$ does play a role.

Several important questions remain. Although the $G_{o}$ 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 Sid-erovski, 2002) are important. Given that nearly 15 different RGS proteins can function as a GTPase-activating protein for $G_{o}$, 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 $G_{o}$ in vitro (Lan et al., 2000). A second question is whether the same enhancement of $\alpha_{2A}$AR 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 $\alpha_{2A}$AR agonist that can selectively activate $G_{o}$ versus $G_{o2}$ or other $G_{i}$ 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 $\alpha_{2A}$ARs on target neurons over $\alpha_{2A}$AR autoreceptors that would decrease NE release. This could be achieved by a “functionally selective” (Urban et al., 2007) $\alpha_{2A}$AR agonist. Second, RGS proteins have been implicated as potential therapeutic targets. Several peptide (Jin et al., 2004; Young et al., 2004; Rooft 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$_{50}$ 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 $G_{o}/G_{S}$ mice but not $G_{o}/G_{S}$ 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$_{50}$ values) calculated for EPI in brain slices from $G_{o}/G_{S}$ 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 $G_{o}/G_{S}$ 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 ($G_{o}/G_{S}$, 74 ± 3.8%; $G_{o}$ litter mate control, 74 ± 4.4%; $G_{o}/G_{S}$, 73 ± 2.8%; $G_{o}$ 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 (Gαq), 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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