RGS Protein Suppression of G\(\alpha_o\) Protein-Mediated \(\alpha_{2A}\) Adrenergic Receptor

Inhibition of Mouse Hippocampal CA3 Epileptiform Activity

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Running title page

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ACSF, artificial cerebral spinal fluid; AR, adrenergic receptor; CA, cornu ammonis; CNS, central nervous system; EPI, epinephrine; GPCR, G-protein coupled receptor; KO, knockout; LTP, long-term potentiation; NE, norepinephrine; RGS, regulator of G-protein signaling; WB-4101, 2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane hydrochloride; WT, wild-type.
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 αAR antagonists atipamezole (8.79), rauwolscine (7.75), WB-4101 (6.87), and prazosin (5.71). Calculated pKb values correlated best with affinities previously determined for the mouse α2AAR subtype (r = 0.98, slope = 1.07). Furthermore, the inhibitory effects of EPI were lost in hippocampal slices from α2AAR-, but not α2CAR-knockout mice. Pretreatment with pertussis toxin also reduced the EPI-mediated inhibition of epileptiform bursts. Lastly, using knock-in mice with point mutations that disrupt RGS binding to Gα 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 to either Gα2^G184S heterozygous (Gα2^+/GS) or control mice (EC50 = 2.5 nM versus 19 nM and 23 nM, respectively). Together, these findings indicate that the inhibitory effect of EPI on hippocampal CA3 epileptiform activity utilizes an α2AAR/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.
Introduction

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 appear 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 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, as well as 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 (LTP), 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 appear to increase seizure susceptibility (Weinshenker and Szot, 2002). While 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 enhancement of LTP (Hopkins and Johnston, 1988) and memory (Devauges 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 α2AARs 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 Gα-GTP and G-βγ subunits is achieved by catalyzing nucleotide exchange on Gα 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 Gα-bound GTP, a step that controls the duration of the signal. The GDP-bound Gα subunit will then reform with the G-βγ heterodimer forming an inactive trimer once again.

For some Gα families (G1o and Gq) 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 G1o and Gq family of G proteins (Clark et al., 2008) and enhance intrinsic GTPase activity of the GTP-bound Gα subunits. This GTPase acceleration attenuates G protein signaling by resetting the Gα subunit to its inactive conformation (Hollinger and Hepler, 2002). Interfering with the activity of RGS proteins allows the Gα 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 $\alpha_2$ARs 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 $\alpha_2$AR knockout and RGS
insensitive Go subunit knock-in mice. Delineating which $\alpha_2$AR and G protein subtype(s) are
involved in attenuating hippocampal epileptiform activity will help 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 Cookston Inc. (Ellisville, MO). All chemical reagents used to make the artificial cerebral spinal fluid (ACSF) were of biological grade from J.T. Baker, Inc. (Phillipsburg, NJ) or Fisher Scientific Co. (Fairlawn, NJ). Isoflurane was purchased from Abbott Diagnostics (Chicago, IL).

Animals. C57BL/6J mice of both sexes were used in the present study. Mice were housed two to four per cage (size 11.5 x 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 four days after arrival (see also below). All protocols described have been approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University (Atlanta, GA), the University of Michigan and the University of North Dakota, 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 (KO) mice. α2A−/− (α2A/α2C; −/−/++) and α2C−/− (α2A/α2C; +/+/−) mice, maintained on a pure C57BL/6J background, were generated at Emory University using heterozygous α2C+/− and α2AC+/− mice obtained from Brian K. Kobilka (Stanford University, Stanford, CA). Genotypes were confirmed by PCR. 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 two to five months. Control animals used in these studies were wild-type (WT) C57BL/6J (α2A/α2C; +/+/++) mice purchased from Jackson Laboratory, Bar Harbor, ME.

**Generation of GαoG184S heterozygous (Gαo+/-GS) knock-in mice.** The original GαoG184S ES cell line, described in Fu et al. (2004, 2006), was developed in a 129-D3 ES cell background which never went germline. Consequently, the GαoG184S mouse strain was constructed from a 129-CJ7 ES line using methods similar to those previously reported for the Gαi2G184S strain (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 (ResGenTM, Invitrogen, Carlsbad, CA). Using those fragments, a targeting construct was prepared in the TKLN vector (Mortensen et al., 1992). First the mutant Gαo exon 5 was produced by mutating the sequence AAAACAACTGGCATCGTAGAAA to AAAACAACTAGTATCGTAGAAA. The bold bases indicate the changed codon (Gly184 to Ser184) and the underline show 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 TKLN to introduce the loxP-flanked neo marker after exon 5, then the right arm genomic fragment from exon 6-8 was cloned 3' of the loxP cassette in a manner similar to that for preparing the Gαi2G184S 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/6NCrl x (C57BL/6J x DBA/2J)F1 mouse blastocysts to generate ES cell-mouse chimeras. After identification of chimeric offspring, the mice were backcrossed onto a CJ7BL/6J background for at least four generations. Only heterozygous offspring of crosses between Gαo+/G184S males and C57BL/6J females (N4-N5) were used in these studies because homozygous GαoG184S/G184S offspring from het x het crosses
were not viable. Control animals used in these studies were WT littermates (+/+) of the Gαo\textsuperscript{G184S} heterozygous (Gαo+/GS) mice.

**Generation of Gαi\textsubscript{2} G184S heterozygous (Gαi\textsubscript{2}+/GS) knock-in mice.** Gαi\textsubscript{2} G184S heterozygous (Gαi\textsubscript{2}+/GS) knock-in mice, maintained on a pure C57BL/6J background (> 10 generations), were generated at the University of Michigan, Ann Arbor, as previously described (Fu et al., 2006). All genotypes were confirmed by PCR. Control animals used in these studies were WT C57BL/6J mice from Jackson Laboratory. Both the Gαo\textsuperscript{G184S} and Gαi\textsubscript{2} G184S heterozygous (+/GS) knock-in mice were reared at the University of Michigan and were confirmed to be pathogen-free prior to their shipping to the University of North Dakota at age one to three months.

**Hippocampal Slice Preparation.** After being deeply anesthetized with isoflurane, mice weighing 16 to 27 g were decapitated and their brains 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 MgSO\textsubscript{4}, 0.5 mM CaCl\textsubscript{2}, 1.25 mM NaH\textsubscript{2}PO\textsubscript{4}, 25 mM NaHCO\textsubscript{3}, 25 mM D-glucose, 11.6 mM sodium ascorbate, and 3.1 mM sodium pyruvate, saturated with 95% O\textsubscript{2}, 5% CO\textsubscript{2}. Using a conventional tissue sectioning apparatus (Stoelting, Wood Dale, IL), the hippocampi were sliced transversely into 500-µm thick sections and transferred to artificial cerebral spinal fluid (ACSF) consisting of 119 mM NaCl, 5 mM KCl, 1.3 mM MgSO\textsubscript{4}, 2.5 mM CaCl\textsubscript{2}, 1 mM NaH\textsubscript{2}PO\textsubscript{4}, 26.2 mM NaHCO\textsubscript{3}, and 11 mM D-glucose, which was continually aerated with 95% O\textsubscript{2}, 5% CO\textsubscript{2}. 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 prior to 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 mL/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 a SZ-61 stereo microscope (Olympus, Melville, NY). Potentials were detected using either an Axoclamp 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 associational connections (Schwartzkroin, 1986). This activity was easily generated by superfusing the slice with ACSF containing 100 μM picrotoxin, a GABA_\text{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 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 long application (data not shown).

Since the α_2AR antagonist rauwolscine also has potent serotonergic 5HT_{1A} receptor-mediated agonist activity (Newman-Tancredi et al., 1998), we substituted 3 μM pindolol (which blocks both βAR and 5HT_{1A} receptors) for timolol (which only blocks βARs) when using this particular α_2AR antagonist.
**Data Analysis.** Epileptiform burst discharge frequencies were visualized in real time (Fig. 1A) while being recorded for subsequent analysis. Post-experiment 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, San Diego, CA) and concentration-response curves were constructed using a non-linear least-squares curve-fitting method. Each curve was fit 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 EC\(_{50}\) 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 (pK\(_b\)) 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 EC\(_{50}\) values were calculated in the presence and absence of a selective α\(_2\)AR 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 non-unity if the 95% confidence interval did not include the value of 1. The pK\(_b\) values of αAR antagonists causing competitive inhibition of the EPI-mediated reduction in burst frequencies were calculated from Schild regression \( x \)-intercepts. Differences in pK\(_b\) values and Schild regression slopes were determined by analysis of covariance with a \( p < 0.05 \) level of probability accepted as significant. EC\(_{50}\) and pK\(_b\) 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 EC₅₀ 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 α₂AR 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 α₂AR Antagonist Atipamezole and Subtype-selective α₂AR Antagonists on the EPI-Mediated Decrease in Burst Discharge Frequencies. Functional determination of equilibrium dissociation constant (Kᵦ) 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 pKᵦ of 8.79 (n = 5) for atipamezole (Fig. 3B) was similar to previously published binding pKᵦ values for the mouse α₂ARs (Link et al., 1992; Chruscinski et al., 1992; see also Table 1). This result suggests that the response is mediated by an α₂AR.

Subtype selective antagonists were then used to determine the specific subtype of α₂AR mediating burst frequency reduction in the mouse hippocampal CA3 region. Apparent pKᵦ values of subtype selective α₂AR competitive antagonists were determined using Schild
regression analysis. Slices pretreated with either prazosin (α2AR-selective), rauwolscine (α2C-AR-selective), or WB-4101 (α2C-AR-selective) produced parallel rightward shifts of the fitted EPI concentration-response curve in all instances (data not shown). For each of these selective α2AR 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 α2AR antagonists were as follows: rauwolscine (7.75, n = 3), WB-4101 (6.87, n = 3), and prazosin (5.71, n = 4) (see Table 1).

α2AR Antagonist Functional Affinity (pK_b) Estimates Correlate to α2AAR Binding (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 α2AR subtype (see also Table 1). For the mouse α2AAR 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 α2BAR, a poor correlation coefficient (r = 0.88) and low slope (slope = 0.40) were observed when comparing our experimental pK_b values with previously published pK_i values (Fig. 4B). Likewise for the mouse α2CAR, a poor correlation coefficient (r = 0.89) and low slope (slope = 0.63) were seen (Fig. 4C). These results suggest that the α2AAR is the predominant subtype mediating the antiepileptic action of EPI in mouse hippocampus.

Effects of EPI on Epileptiform Activity in Slices from α2A- and α2C-AR-KO Mice. To confirm our pharmacological results, we examined the effects of EPI on hippocampal CA3
epileptiform activity in α2AR- and α2CAR-KO mice. As illustrated in Fig. 5, EPI was applied in increasing concentrations to hippocampal brain slices prepared from either α2AR- 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 α2AR-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 α2AR Antagonist Oxymetazoline on the EPI-Mediated Decrease in Burst Discharge Frequencies in α2CAR-KO Mice. To further evaluate a potential role for α2BARs and confirm that the response was primarily an α2AR response, the selective α2AR 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 pKi of 7.49 matched closely to our pKb value, while the α2BAR and α2CAR reported pKi’s 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 one. These results further confirm that this response is primarily mediated by an α2AR.

Effects of Pertussis Toxin (PTX) on EPI-Mediated Inhibition of CA3 Epileptiform Activity. PTX blocks the receptor-mediated activation of G\textsubscript{i/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 slices treated with 5 μg/ml PTX for 7 to 8-h. As illustrated in Fig. 7, the mean concentration-response curve for non-treated control slices was fit best by a unity-slope sigmoidal model with a calculated EC\textsubscript{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\textsubscript{i} or G\textsubscript{o} proteins, and not G\textsubscript{s} or G\textsubscript{q} proteins.

**EPI-Mediated Inhibition of CA3 Epileptiform Activity in Slices from G\textsubscript{o}\textsubscript{G184S Heterozygous (G\textsubscript{o}+/GS) and G\textsubscript{i2}G184S Heterozygous (G\textsubscript{i2}+/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 G\textsubscript{o} subunit mutation (G184S) that renders G\textsubscript{o} and G\textsubscript{i2} proteins incapable of binding to the RGS protein. This results in the loss of RGS-mediated inhibition of the G\textsubscript{o} and G\textsubscript{i2} protein and enhances G\textsubscript{o}-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, G\textsubscript{o}+/GS or G\textsubscript{i2}+/GS slices were pretreated with the GABA blocker picrotoxin, βAR blocker timolol, and NE transporter re-uptake 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 G\textsubscript{o} mice, with an EC\textsubscript{50} of 2.5 ± 0.9 nM (n = 23 slices) versus littermate control mice (EC\textsubscript{50} = 19 ± 5 nM, n = 21 slices) (Fig. 8A). In contrast, there was no significant difference in G\textsubscript{i2} mice (EC\textsubscript{50} = 19 ± 5 nM, n = 32 slices) compared to the WT controls (EC\textsubscript{50} = 23 ± 7 nM, n = 22 slices) (Fig. 8B). These
results indicate the EPI-mediated inhibition of mouse hippocampal CA3 epileptiform activity involves a \( \text{G}_{\alpha_0} \) 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 (β, α₁, and α₂) can be antiepileptic, but the most consistent findings show that α₂AR agonists are generally anticonvulsant and selective α₂AR antagonists 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 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 Gᵢₒ-type G protein. Furthermore, using RGS-insensitive Gα subunit mutant knock-in mice, we show that endogenous RGS protein action on Gαₒ strongly suppresses this signal, implicating Gαₒ as a mediator of the response. In contrast, Gαᵢ₂ appears 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, α₂AAR, and α₂C-AR-KO mice showed that the proconvulsant effects of α₂AR agonists were mediated by the α₂AAR autoreceptor, which decreases NE release, while the anticonvulsant effects of α₂AR 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 (antagonist $pK_b$) and genetic ($\alpha_{2A}$AR- and $\alpha_{2C}$AR-KO) approaches. Despite expression of the $\alpha_{2C}$AR in hippocampus, it does not appear to contribute at all to the antiepileptiform activity of EPI and NE (Fig. 5). Similarly, the $\alpha_{2B}$AR does not seem to play a role (Fig. 6). Neither were $\alpha_1$ARs involved in this particular response (Fig. 3, 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 $\alpha_{2}$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 $G_{i/o}$ 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 $K^+$ (GIRK) currents, etc. (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 have been implicated as potential drug targets (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 as 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 $G_{i/o}$ subtype(s) can mediate EPI’s effect, mice with knock-in mutant RGS-insensitive $G_{o_1}$ or $G_{o_2}$ 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 α and βγ subunits derived from that G protein. The heterozygous Gαo RGS-insensitive (Gαo+/G184S) knock-in animals showed a 7-fold leftward shift of the EPI dose response curve (2.5 nM versus 19 nM), while there was no significant difference in potency between the heterozygous Gα2 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 where even half of the G protein removed from this suppression could produce a marked increase in signaling. Previous studies with the Gα2G184S 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 Gαo subtype of G i/o proteins is involved in the signaling mechanism while Gα2 appears not to be. At this stage, we cannot rule out a contribution from other pertussis toxin sensitive G proteins such as Gαi1 or Gαi3, but the evidence clearly indicates that Gαo does play a role.

Several important questions remain. While the Gαo 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 Gαo, it may be difficult to establish which one(s) are involved. Furthermore, it is possible 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 since they are relatively selective for Gαo 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 α2AR agonist that can selectively activate Gαo versus Gαi2 or other Gαi 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 α2ARs on target neurons over α2AR autoreceptors that would decrease NE release. This could be achieved by a “functionally selective” (Urban et al., 2007) α2AR 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 non-peptide (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 could potentially reduce side effects on treatment with α2AR selective agonists in epilepsy patients.

In summary, we have defined the receptor (α2AR), a G protein (Gαo), 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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References


Footnotes

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Legends for Figures

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 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 ten bursts (0.067 Hz) in control ringer to seven (0.047 Hz) in 30 nM EPI, three (0.020 Hz) in 300 nM EPI, and one (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. Insert, concentration-response curve derived from the frequency histogram. Data points were plotted as percent of maximal inhibition (decrease in epileptiform burst frequency) and the curve 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.

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 (EC_{50} 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 percent 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.

Fig. 3. Schild regression analysis using the selective α2AR antagonist atipamezole. A, consecutive EPI concentration-response curves demonstrate a concentration-dependent effect of the selective α2AR antagonist, atipamezole, on the EPI-mediated inhibition of hippocampal CA3 epileptiform activity in brain slices from WT mice. Pretreatment with 3 nM (○), 10 nM (■), and 30 nM (□) of this antagonist produced consecutive parallel rightward shifts of the EPI curve that were significantly different from control (●) [EC50 = 76 ± 3, 218 ± 30, and 762 ± 274, respectively, versus 34 ± 12 nM for control]. B, using dose ratios calculated from individual experiments illustrated in A, a Schild plot was created generating a regression slope equaling 1.06 ± 0.12 and an x-intercept correlating to a pKb value of 8.79, n = 5 animals (see Table 1).

Fig. 4. Correlation between the functional affinity estimates (pKb) to the equilibrium dissociation constants (pKi) for various selective α2AR antagonists. Using the pKb and pKi values from Table 1, correlation analyses were performed for A, the α2AAR, B, the α2BAR, and C, the α2CAR.

Fig. 5. Effects of EPI on hippocampal CA3 epileptiform activity in brain slices from α2AAR- and α2CAR-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 μM picrotoxin, 30 μM timolol and 0.5 μM desipramine. Concentration-response curves for EPI were plotted as a percent of decrease (reduction) in epileptiform burst frequency. For the α2AAR-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 α₂CR-KO mice was fit best by a nonvariable sigmoidal model with a calculated EC₅₀ value of 37 ± 12 nM, which was not significantly different than 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 α₂CR-KO mice was 64 ± 3.9 %, which was significantly different from the 8.7 ± 3.3 % inhibition for EPI in slices from α₂AR-KO mice.

**Fig. 6.** Schild regression analysis using the selective α₂AR ligand oxymetazoline in slices from α₂CR-KO mice. A, consecutive EPI concentration-response curves demonstrate a concentration-dependent effect of the selective α₂AR ligand, oxymetazoline, on the EPI-mediated inhibition of hippocampal CA3 epileptiform activity in brain slices from α₂CR-KO mice. Pretreatment with 100 nM (○), 300 nM (■), and 1000 nM (□) of this antagonist produced consecutive parallel rightward shifts of the EPI curve that were significantly different from control (●) [EC₅₀ = 205 ± 48 nM, 738 ± 267 nM, 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 pKᵦ value of 7.50, n = 7 animals. This pKᵦ value matched the binding affinity of oxymetazoline (pKᵦ = 7.49) for the mouse α₂AR, but not the mouse α₂BAR (pKᵦ = 5.92) (Chruscinski et al., 1992) or mouse α₂CR (pKᵦ = 6.96) (Link et al., 1992).

**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 percent of decrease (reduction) in epileptiform burst frequency. The mean
concentration-response curve for non-treated control slices was fit best by a nonvariable sigmoidal model with a calculated EC₅₀ 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 Gαₒ+/GS mice, but not Gαᵢ₂+/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 non-variable sigmoidal curve. **A,** There was a significant difference in the potencies (EC₅₀ values) calculated for EPI in brain slices from Gαₒ+/GS mice (□) (2.5 ± 0.9 nM, n = 23 slices from 6 animals) versus littermate 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 Gαᵢ₂+/GS mice (□) (19 ± 5 nM, n = 32 slices from 6 animals) compared to WT control mice (●) (23 ± 7 nM, n = 22 slices from 8 animals). There was no significant difference in the efficacy of EPI between these four groups (Gαₒ+/GS, 74 ± 3.8 %; Gαₒ littermate control, 74 ± 4.4 %; Gαᵢ₂+/GS, 73 ± 2.8 %; Gαᵢ₂ WT control, 75 ± 3.6 %).
Tables

TABLE 1

Comparisons of experimental functional $pK_b$ values to binding affinity $pK_i$ values for selective $\alpha$AR antagonists for mouse $\alpha_2$AR subtypes. $pK_b$ values represent the negative logarithm of the $K_b$ and are expressed as the mean. Schild regression slopes are expressed as the mean slope $\pm$ S.E. and were determined in three to five separate experiments using brain slices from WT mice. Reported $pK_i$ values are from binding affinity studies using recombinant mouse $\alpha_2$AR clones expressed in COS-7 cells.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>$pK_b$</th>
<th>Slope</th>
<th>$\alpha_2A$AR</th>
<th>$\alpha_2B$AR</th>
<th>$\alpha_2C$AR</th>
</tr>
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<tbody>
<tr>
<td>Atipamezole</td>
<td>8.79</td>
<td>1.06 $\pm$ 0.12</td>
<td>9.07$^a$</td>
<td>8.30$^b$</td>
<td>8.80$^a$</td>
</tr>
<tr>
<td>Rauwolscine</td>
<td>7.75</td>
<td>1.01 $\pm$ 0.07</td>
<td>7.27$^a$</td>
<td>8.14$^b$</td>
<td>9.10$^a$</td>
</tr>
<tr>
<td>WB-4101</td>
<td>6.87</td>
<td>0.89 $\pm$ 0.08</td>
<td>6.58$^a$</td>
<td>7.15$^b$</td>
<td>8.11$^a$</td>
</tr>
<tr>
<td>Prazosin</td>
<td>5.71</td>
<td>0.97 $\pm$ 0.06</td>
<td>5.67$^a$</td>
<td>7.23$^b$</td>
<td>7.01$^a$</td>
</tr>
</tbody>
</table>

* $pK_b$ was calculated using a single 10 $\mu$M concentration of JP-1302.

N.A., not available.

$^a$ Link et al. (1992).

$^b$ Chruscinski et al. (1992).
Figure 3

A

% Reduction in Burst Frequency

Log M [Epinephrine]

B

Log [Dose Ratio - 1]

Log M [Atipamezole]
Figure 5

% Reduction in Burst

Frequency

Log M [Epinephrine]

-10 -9 -8 -7 -6 -5 -4

0 25 50 75 100

○ α₂A -/-

● α₂C -/-