Amperometric Measurement of Glutamate Release Modulation by Gabapentin and Pregabalin in Rat Neocortical Slices: Role of Voltage-Sensitive Ca\(^{2+}\) \(\alpha_2\delta-1\) Subunit

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ABSTRACT

Gabapentin (GBP; Neurontin) and pregabalin (PGB; Lyrica, S\(^-\)+)-3-isobutylgaba) are used clinically to treat several disorders associated with excessive or inappropriate excitability, including epilepsy; pain from diabetic neuropathy, postherpetic neuralgia, and fibromyalgia; and generalized anxiety disorder. The molecular basis for these drugs’ therapeutic effects are believed to involve the interaction with the auxiliary \(\alpha_2\delta\) subunit of voltage-sensitive \(\text{Ca}^{2+}\) channel (VSCC) translating into a modulation of pathological neurotransmitter release. Glutamate as the primary excitatory neurotransmitter in the mammalian central nervous system contributes, under conditions of excessive glutamate release, to neurological and psychiatric disorders. This study used enzyme-based microelectrode arrays to directly measure extracellular glutamate release in rat neocortical slices and determine the modulation of this release by GBP and PGB. Both drugs attenuated \(\text{K}^+\)-evoked glutamate release without affecting basal glutamate levels. PGB (0.1–100 \(\mu\)M) exhibited concentration-dependent inhibition of \(\text{K}^+\)-evoked glutamate release with an IC\(_{50}\) value of 5.3 \(\mu\)M. \(\text{R}-(\text{--})\)-3-Isobutylygaba, the enantiomer of PGB, did not significantly reduce \(\text{K}^+\)-evoked glutamate release yet inhibited this release in wild-type mice. The results show that GBP and PGB attenuated stimulus-evoked glutamate release in rodent neocortical slices and that the \(\alpha_2\delta-1\) subunit of VSCC appears to mediate this effect.

Introduction

Several neurological and psychiatric disorders characterized by excessive or dysfunctional neurotransmitter release are routinely treated with gabapentin (GBP; Neurontin, 1-(aminomethyl)cyclohexanecarboxylic acid) and pregabalin (PGB; Lyrica, S\(^-\)+)-3-isobutylgaba, S\(^-\)+)-4-amino-3-(2-methylpropyl)butanoic acid) (Dooley et al., 2007). Although multiple mechanisms of action have been proposed historically to account for the preclinical and clinical profiles of these drugs, there is increasing evidence for a significant role of the auxiliary \(\alpha_2\delta\) subunit of voltage-sensitive \(\text{Ca}^{2+}\) channel (VSCC) (Taylor et al., 1998, 2007). The binding of these ligands to the \(\alpha_2\delta\) subunit is believed to be the source of their efficacy in treating epilepsy; pain from diabetic neuropathy, postherpetic neuralgia, and fibromyalgia; and generalized anxiety disorder. With the recent availability of transgenic mice with point mutations of the \(\alpha_2\delta\) subunit (i.e., \(\alpha_2\delta-1\) and \(\alpha_2\delta-2\) subtypes) (Bian et al., 2006, 2008), preclinical experiments can be designed to test for altered neurochemical and behavioral effects of GBP and PGB (Field et al., 2006).

In the present study, we used enzyme-based microelectrode arrays (MEAs) that have micrometer-sized platinum recording sites with sampling rates of >1 Hz. These MEAs were developed in response to the limitations of other techniques or devices used to measure neurotransmitters (e.g., microdialysis/perfusate sampling coupled to high-performance liquid chromatography) (Barnes et al., 1988; Shinohara et al., 1998; Burmeister et al., 2000). A drawback of microdialysis or perfusate sampling techniques is that they sample from a large area (Borland et al., 2005) and at relatively slow (minutes) sampling...
rates. Given the rapid nature of neurotransmission of chemical messengers such as glutamate, faster sampling rates and smaller sampling areas should be beneficial. Enzyme-coated MEAs have been characterized extensively in the brains of anesthetized and behaving animals to measure glutamate (Burmeister et al., 2002; Binns et al., 2005; Day et al., 2006; Nickell et al., 2006; Rutherford et al., 2007; Parikh et al., 2010) but not in brain slices.

We used these MEAs to directly measure $K^+$-evoked (extracellular) glutamate release in rat neocortical slices and to determine the modulation of this release by GBP and PGB. As the primary excitatory neurotransmitter in the mammalian central nervous system, glutamate often has been associated with a variety of pathological conditions (Meldrum, 2000), several of which are responsive to $\alpha_2\delta$ ligands such as PGB. A reduction of excessive glutamate release by $\alpha_2\delta$ ligands conceivably translates into clinically relevant therapeutic effects, especially considering the experimental evidence supporting a relationship between $\alpha_2\delta$-subunit binding and the modulation of processes subserving neurotransmitter release (Dooley et al., 2007).

An additional aspect of this study was to assess the effects of PGB on $K^+$-evoked (extracellular) glutamate release in neocortical slices from wild-type and $\alpha_2\delta$-1 mutant mice. The $\alpha_2\delta$-1 transgenic mice have a point mutation (namely, R217A) that markedly reduces $[^3H]$GBP and $[^3H]$PGB binding in central nervous system regions (e.g., neocortex) known to preferentially express the $\alpha_2\delta$-1 protein (Bian et al., 2006; Field et al., 2006).

Materials and Methods

Animals. Male rats [Sprague-Dawley, 2–8 weeks old; Harlan, Indianapolis, IN] and male mice [wild-type and mutant $\alpha_2\delta$-1 R217A (Bian et al., 2006; Field et al., 2006), 2–5 months old; Charles River Laboratories, Inc., Wilmington, MA] were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility according to the standards outlined in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

Animals were under a 12-h light/dark cycle, had ad libitum access to food and water, and were maintained for a minimum of 5 days before euthanasia by decapitation. The brains were removed by blunt dissection and placed in ice-cold buffer until slice preparation. Before euthanasia by decapitation. The brains were removed by blunt dissection and placed in ice-cold buffer until slice preparation. Before euthanasia by decapitation. The brains were removed by blunt dissection and placed in ice-cold buffer until slice preparation. Before euthanasia by decapitation. The brains were removed by blunt dissection and placed in ice-cold buffer until slice preparation. Before euthanasia by decapitation. The brains were removed by blunt dissection and placed in ice-cold buffer until slice preparation.

Glutamate Release Measurements. Neocortical slices from rats and mice were prepared using standard protocols (Hascup et al., 2007). In brief, coronal slices (0.35–0.4 mm in thickness), including the Animal Care and Use Committee of the University of Kentucky.

Results

The effects of GBP and PGB on resting glutamate levels were evaluated in initial experiments. Neither drug (0.1–100 $\mu$M) altered basal glutamate levels in neocortical slices (data not shown).

In the brains of anesthetized animals, delivery of high $K^+$ to stimulate glutamate release has been performed with local, pressure-ejected administration (Burmeister et al., 2002; Day et al., 2006; Quintero et al., 2007; Stephens et al., 2009). Meanwhile, studies in brain slices permit the use of two methods of delivering high $K^+$ solutions to activate neural networks: local delivery and superfusion. Local stimulation, using pressure delivery, produces a comparable stimulus to
those used in previous studies with anesthetized animals. An additional benefit of brain slice recordings is the flexibility to also use superfusion of high K⁺ to evoke release. This sustained depolarization resembles prolonged or excessive excitability—a condition that characterizes some neurological disorders such as anxiety. We used both stimulation methods here to characterize the effects of αδ subunit ligands and to compare glutamate measurements with MEAs to those of previous studies.

The repeated pressure-ejection delivery of 70 mM K⁺ solution yielded similar-sized glutamate signals with a mean amplitude of 3.9 ± 0.8 μM (Fig. 1). In the presence of GBP (100 μM), the mean amplitude was decreased significantly by 46% to 2.1 ± 0.7 μM (Fig. 1).

Because GBP was confirmed to modulate K⁺-evoked glutamate release, the more recently developed αδ ligand PGB was chosen for testing in additional experiments. PGB (100 μM) attenuated pressure-ejection delivery of 70 mM K⁺ solution (5.7 ± 1.5 μM glutamate, pre-PGB versus 1.7 ± 1.2 μM glutamate, post-PGB; t(4) = 3.59, p = 0.023). We then transitioned to a paradigm of using repeated stimulation with superfused 70 mM K⁺ (S₁, S₂). The S₂/S₁ ratio of control glutamate signals in rat neocortical slices was 0.97 (Figs. 2A and 3A); this ratio was markedly reduced by 78% to 0.21 by GBP (100 μM) (Figs. 2B and 3A). Other S₂/S₁ ratios for PGB include 0.80 (nonsignificant, 14% inhibition) at 0.1 μM, 0.60 (nonsignificant, 38% inhibition) at 1 μM, and 0.44 (54% inhibition) at 10 μM (Fig. 3A); an IC₅₀ value of 5.3 μM was determined from the concentration-effect relationship (Fig. 3B). The enantiomer of PGB, R-IBG (100 μM), gave an S₂/S₁ ratio of 0.70 (nonsignificant, 28% inhibition), contrasting sharply with the effect (78% inhibition) of an equimolar concentration of PGB (Fig. 3A).

PGB and the endogenous amino acid L-isoleucine are sub-

![Fig. 1. Effect of GBP (100 μM) on K⁺-evoked glutamate release from rat neocortical slices. A, glutamate release evoked by repeated pressure-ejection delivery of 70 mM K⁺ solution (arrowheads on abscissa) in the absence (first three traces) and presence of GBP (last three traces). B, amplitude of K⁺-evoked glutamate release (as derived from A) was decreased by GBP. Values given are X ± S.E. (n = 6). The paired t statistic gave t(5) = 2.930 (p = 0.0326). A significant difference from the control value is indicated by an asterisk (*, p = 0.05).](image)

strates for the system L-amino acid transporter, and both have a similar nanomolar affinity for the αδ ligand binding site on the αδ subunit. The S₂/S₁ ratio associated with the K⁺-evoked glutamate signals in the presence of L-isoleucine (100 μM) was 0.84 (nonsignificant, 13% inhibition), yet this compound reduced the effect (78% inhibition) of GBP (100 μM) as indicated by the S₂/S₁ ratio of 0.59 (nonsignificant, 39% inhibition) (Fig. 3A).

An action of GBP and PGB at the αδ-1 subtype rather than the αδ-2 subtype has been proposed to account for the therapeutic effects of these drugs (Bian et al., 2006, 2008; Field et al., 2006). With neocortical slices from the wild-type and αδ-1 transgenic mice (Bian et al., 2006; Field et al., 2006), PGB (100 μM) significantly decreased K⁺-evoked glutamate signals (Fig. 4, A and B) in wild-type mice by 36% [S₂/S₁ ratio of 0.92 (control) versus 0.59 (PGB); n = 13 and 14, respectively] (Fig. 5); in the αδ-1 transgenic mice, the glutamate signals were unchanged by this drug [S₂/S₁ = 0.74 (control) versus 0.63 (PGB); n = 9 each] (Fig. 4, C and D).

### Discussion

Aberrant glutamate neurotransmission is linked to a variety of neurological and psychological disorders. Thus, identifying mechanisms that could modulate abnormal glutamate release may provide an avenue for developing new therapeutics for modulating glutamate signaling. Here, we used enzyme-based MEAs to directly measure extracellular glutamate and observed that both GBP and PGB attenuated the K⁺-evoked glutamate release.

GBP has been used as an antiepileptic, but its precise mechanism of action is unknown (Taylor et al., 2007). To help address this, we stimulated the neural network and measured synaptic spillover of glutamate using a technique that
Fig. 3. Effects of PGB (0.1–100 μM), R-(−)-3-isobutyrgaba (100 μM), and L-isoleucine (100 μM) to inhibit K⁺-evoked glutamate release in rat neocortical slices. A, concentration–effect relationship of PGB and inactivity of R-(−)-3-isobutyrgaba, L-isoleucine, and PGB (100 μM) and L-isoleucine (100 μM) combination after repeat superfusion with 70 mM K⁺ (S₁, S₂) for 50 s. Substances were present 15 min before S₂. Values given are X ± S.E. (n = 7). Analysis of variance of S₂/S₁ values for control and PGB concentrations gave F(4,30) = 5.17 (p = 0.003). A significant difference from the control value is indicated by an asterisk (*, p ≤ 0.05 and ***, p ≤ 0.001). The S₂/S₁ ratios obtained for the other substances, including the PGB and L-isoleucine combination, were not significantly different from the control value. B, the transformed data from A depict inhibition (%) by PGB relative to the mean control S₂/S₁ ratio of 0.97 normalized to 1.0; the corresponding IC₅₀ value was 5.3 μM.

we have used previously in anesthetized animals of locally delivering high K⁺ solution to evoke depolarization and produce a release of glutamate (Burmeister et al., 2002; Day et al., 2006). We observed in these brain slices an attenuation of glutamate release by GBP after locally delivering high K⁺, an effect on neurotransmitter release that is repeatedly observed after stimulus delivery (Dooley et al., 2000a,b, 2002).

GBP is related structurally to GBP but with greater reported efficacy in clinical studies (Taylor et al., 2007). Using these MEAs, we used a whole slice superfusion of high K⁺ to stimulate neurotransmitter release similar to Dooley et al. (2000b). We have observed that this type of depolarization evokes glutamate release that is calcium dependent (J. E. Quintero and G. A. Gerhardt, unpublished observations). The dose-dependent inhibition of K⁺-evoked glutamate release by PGB resulted in an IC₅₀ value of 5.3 μM compared with an IC₅₀ value of 11.8 μM of K⁺-evoked [³H]norepinephrine release (Dooley et al., 2002).

The magnitude of the attenuation of evoked glutamate release was larger than those reported in some studies that have examined the effect of GBP or PGB on stimulus-evoked neurotransmission (Dooley et al., 2000a,b, 2002; Brown and Randall, 2005). One apparent factor that may influence the effectiveness of these compounds is the type of stimulus, such that GBP and PGB may exert an effect on the prolonged, depolarization-induced neurotransmitter release that more closely resembles hypereexcitability as found in pathological states rather than normal physiological neurotransmission (Dooley et al., 2000a, 2007; Maneuf et al., 2001). In addition, some of the GBP or PGB effects on stimulus-evoked neurotransmitter release may have been diluted in studies on slices or synaptic endings where the whole chamber perfusate is sampled (Dooley et al., 2000a; Fink et al., 2000, 2002) versus the limited focal area sampled by the 50 × 150 μm size of these MEAs in slices. For example, with the high-resolution technique of whole-cell patch clamp in cortical slices, GBP and PGB show an effect as high as ~50% on parameters related to glutamate neurotransmission (Cunningham et al., 2004). Nonetheless, the effect of GBP and PGB on neurotransmitter release remains controversial given reports that GBP and PGB have no effect on K⁺-evoked glutamate release from human synaptosomes (Brawek et al., 2009) or that the GBP and PGB effect may be linked to the trafficking of calcium channels to the cell surface (Hendrich et al., 2008; Mich and Horne, 2008; Bauer et al., 2009; Thorpe and Offord, 2010). The full effect of these ligands may result from both altering calcium channel trafficking and more rapid modulation of synaptic function (Taylor, 2009).

Meanwhile, the pharmacological effects of PGB are stereo-selective, and this was borne out by the nonsignificant changes to the S₂/S₁ ratio with the enantiomer R-IBG. The α amino acids L-isoleucine and L-leucine have been proposed as potential endogenous ligands for the α₅β subunit (Thurlow et al., 1993). Although L-isoleucine did not produce a significant change to K⁺-evoked glutamate release, L-isoleucine did inhibit the PGB attenuation of K⁺-evoked glutamate release similar to what had been described previously with L-isoleucine and GBP in cortical brain slices (Cunningham et al., 2004). A more complex role in neurotransmission and GBP and PGB effectiveness may be the case for these α amino acids where these endogenous ligands may act as “positive modulators required for full functionality of the α₅β subunit” (Hendrich et al., 2008).

In a previous study, Wang et al. (1999) showed that the arginine at position 217 in the α₅ region of the α₅β subunit is critical for GBP binding. Subsequently, in a R217A knockin mouse that was developed, [³H]GBP and [³H]PGB binding to neocortical membranes was greatly reduced in R217A mice compared with that in wild-type mice (Bian et al., 2006; Field et al., 2006). Accordingly, in slices from R217A mice, we concluded that a functional α₅β subunit is necessary for GBP to attenuate K⁺-evoked glutamate release. Although the mean S₂/S₁ ratio (0.74) in slices from the R217A mice was lower than the S₂/S₁ ratio in slices from the wild-type mice (0.92), the means were not significantly different. However, we cannot rule out the possibility of a change in excitability properties of the neurons and synapses in these animals given that the α₅β subunit is critical for normal synapse formation or function (Eroglu et al., 2009).

In summary, we showed that GBP and PGB can modulate stimulus-evoked glutamate release in rat neocortical brain slices and that the α₅β subunit of VSCC is involved in the inhibitory effects of these ligands. This ability to modulate excitatory neurotransmitter release may explain, in
part, the efficacy of these molecules in the clinic. The application of the slice recording methodology coupled to the MEA recording technology establishes a new means to measure neurotransmitter release.

Authorship Contributions

Participated in research design: Quintero, Dooley, Pomerleau, Huettl, and Gerhardt.

Conducted experiments: Quintero.

Contributed new reagents or analytic tools: Gerhardt.

Performed data analysis: Quintero, Dooley, Pomerleau, and Gerhardt.

Wrote or contributed to the writing of the manuscript: Quintero, Dooley, Pomerleau, Huettl, and Gerhardt.

Other: Gerhardt directed research efforts.

Fig. 5. The point mutation R217A prevents PGB from attenuating K+-evoked glutamate release in slices. The $S_2/S_1$ ratio derived from repeated K+ superfusion of slices was decreased by PGB. A main effect of PGB treatment was identified with a two-way analysis of variance ($F(1,41) = 5.57$ ($p = 0.023$)), and a post hoc Bonferroni test revealed a significant effect ($p < 0.05$) of PGB only in slices from wild-type animals. A significant difference from the control value is indicated by an asterisk ($*, p \leq 0.05$). Values given are $X \pm S.E.$

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