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GABA Synapses and the Rapid Loss of Inhibition to Dentate Gyrus Granule Cells after Brief Perforant-Path Stimulation

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Summary: *Purpose:* To study the pharmacologic and synaptic basis for the early loss of paired-pulse inhibition that occurs in the perforant-path stimulation model of status epilepticus.

Methods: Hippocampal slices were prepared from male Wistar rats. Test paired pulses (20- to 50-ms interstimulus interval) of the perforant path were used before and after an abbreviated period of perforant-path stimulation (1–5 min; 2-Hz continuous with 20 Hz of 10 s/min pulses) while either recording field potentials from the dentate gyrus granule cell layer or directly measuring whole-cell patch-clamp currents from granule cells. Paired-pulse field recordings also were obtained during perfusion of the γ-aminobutyric acid $(GABA)_A$ antagonist bicuculline.

Results: Prolonged loss of paired-pulse inhibition occurs after brief (<5 min) perforant-path stimulation in vitro (similar to results in vivo) with the paired-pulse population spike amplitude ratio (P2/P1) increasing from a baseline of 0.53 ± 0.29 to 1.17 ± 0.09 after perforant-path stimulation ($p < 0.05$). After perfusion with the $GABA_A$ antagonist, bicuculline, the P2/P1 ratio also increased from a baseline of 0.52 ± 0.16 to 1.15 ± 0.26 ($p < 0.05$). After 1–2 min of perforant-path stimulation, a $22 \pm 6\%$ ($p < 0.05$) decrease occurred in the P2/P1 amplitude ratio of paired-pulse evoked inhibitory postsynaptic currents.

Conclusions: Similar to in vivo, loss of paired-pulse inhibition occurs with brief perforant-path stimulation in vitro. $GABA_A$ antagonism causes a similar loss of paired-pulse inhibition, and the effects of perforant-path stimulation on postsynaptic inhibitory currents also are consistent with the involvement of $GABA_A$ synaptic receptors. The findings suggest that loss of inhibition at GABA synapses may be an important early event in the initiation of status epilepticus. **Key Words**: Epilepsy—Status epilepticus—$GABA_A$ receptors—Paired-pulse inhibition—Hippocampus.

Status epilepticus (SE) affects >100,000 persons per year in the United States, with >20,000 deaths per year (1). Significant pharmacoresistance to benzodiazepines (BZDs) develops as seizure activity progresses, making treatment more difficult (2), and this may contribute to the significant increases in mortality noted for SE with duration >1 h. The rapid loss of BZD inhibition as seizure activity persists (3–5) suggests that γ-aminobutyric acid (GABA)ergic mechanisms are involved early during SE.

Perforant-path stimulation (PPS) is a reliable animal model that effectively induces SE in rats by 30 min of stimulation (6). GABAergic inhibition, as assessed by response to BZDS, clearly is diminished in this model by 40 min and severely reduced by 70 min (7). Unlike many other animal models of epilepsy, the PPS model permits a convulsant stimulus that can be titrated according to the duration of stimulation. Previously we described a prolonged loss of paired-pulse inhibition lasting >40 min after a very brief period of PPS (<3 min), insufficient to produce prolonged seizure activity (8). This suggested that alterations in inhibition may occur quite early during SE, are important in an initiation phase of SE (9), and may be a significant component in the transition from single seizures to self-sustaining SE.

Because in vivo recordings often are limited to field recordings (especially for prolonged evaluation), this study relates a frequently used physiological index in vivo, paired-pulse inhibition, to the in vitro setting, where more thorough evaluation of mechanism is possible. To explore further the loss of paired-pulse inhibition with brief PPS in vivo, we assessed pharmacologic and synaptic mechanisms by using the hippocampal slice preparation with stimulation of the perforant path in vitro. We find a similar loss of paired-pulse inhibition with brief PPS in vitro, and this loss of paired-pulse inhibition can be explained by effects on GABA synapses. The association of loss of paired-pulse inhibition in vitro with attenuation of GABAergic inhibition likely has implications for loss of paired-pulse inhibition in vivo.

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METHODS

Male Wistar rats older than 4 weeks were anesthetized with halothane, decapitated, and the brains were placed in ice-cold artificial cerebrospinal fluid (aCSF) containing (in m*M*): 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 D-glucose, 1 pyruvate, and 0.3 ascorbic acid (Sigma), at pH 7.3. Coronal sections (Leica VT1000S, Bannockburn, IL, U.S.A.) of 500 μm thickness (for field potentials) or 350 μm (for whole-cell patch-clamp) were held in a chamber at room temperature, bubbled with 95% O_2 and 5% CO_2 until transfer to a chamber perfused continuously with 33–35°C aCSF, where recordings from the dentate gyrus granule cell layer (field potentials) or somata of visualized granule cells (whole-cell patch-clamp) were made by using an Axoclamp-2B amplifier (Axon Instruments, Foster City, CA, U.S.A.). For experiments involving paired-pulse field recordings, aCSF $MgCl_2$ levels were reduced to 0.5–1 m*M*, and $CaCl_2$ was increased to 2.5 m*M*.

Electrode solutions for field recordings consisted of aCSF. Electrode solutions for evoked inhibitory postsynaptic currents (eIPSCs) contained (m*M*): 130 Cs-gluconate, 10 CsCl, 2 $MgCl_2$, 10 Hepes, 0.05 EGTA, 5 TEA-Cl, and 2 Mg-ATP. The holding potential for evoked events was 0 mV. Electrode solutions were titrated to pH 7.25 with osmolarity of 280–300 mOsm. Cs-gluconate blocks potassium channels, including postsynaptic $GABA_B$-mediated currents. Electrode resistance was 4–6 MΩ for both field and whole-cell patch-clamp experiments, and recordings with series resistance changes exceeding 50% were excluded.

Test paired pulses were delivered via the perforant path (pulses, 50–150 V; 0.1–0.5 ms; interstimulus interval, 20–50 ms) and were used to generate action potential–mediated population spikes for field recordings or eIPSCs for whole-cell patch-clamp recordings. Eliciting paired-pulse inhibition was much less reliable in vitro than in vivo (probably because of disruption of inhibitory inputs on to granule cells with slice preparation). Therefore the pulse stimulus intensity and/or duration were increased until maximal paired-pulse inhibition was obtained. Occasionally, slight adjustments in interstimulus interval ~40 ms were necessary to maximize paired-pulse inhibition, more commonly with field recordings. Paired-pulse responses have been characterized as involving an early inhibitory phases most evident at short interstimulus intervals <30 ms and a late inhibitory phase prominent at interstimulus intervals >150 ms (with an intervening period of facilitation) (10–12). It is reasonable to assume that the slight adjustments in interstimulus interval used to obtain paired-pulse inhibition in our experiments still measure the early inhibitory ($GABA_A$) component. Perforant-path stimulation (PPS) consisted of 2-Hz continuous and 20 Hz for 10 s/min pulses for 1–5 min. Stimulating electrodes were bipolar side-by-side with 75-μm tip separation and 0.5–1.5 MΩ resistance (Micro Probe, Potomac, MD, U.S.A.).

Drugs

Bicuculline (Sigma), 1–20 μM, was perfused for selected paired-pulse field recording experiments involving $GABA_A$ antagonism. We adjusted the concentration to avoid spontaneous or prolonged excitatory activity from electrical stimulation that would obscure interpretation of paired-pulse responses.

Data acquisition and analysis

We used 3-kHz low-pass filtering with 10-kHz digitizing (Digidata 1200; Axon Instruments) and event detection and analysis with AxoScope (Axon Instruments).

RESULTS

PPS causes a rapid loss of paired-pulse inhibition in vivo and in vitro in the dentate gyrus

Although PPS for 30 min causes self-sustaining SE (6), we have noted that stimulation for much briefer periods of 1–3 min leads to loss of paired-pulse inhibition of the population spike recorded in the dentate gyrus in vivo (Fig. 1). The loss of inhibition as assessed by paired-pulse responses lasted 43 ± 15 min with ≤3 min PPS (8) and lasted 38 ± 22 min, with only 1 min of PPS. All values are mean ± SD. With in vitro PPS of 1- to 5-min duration, field recordings from the granule cell layer showed loss of paired-pulse inhibition (Fig. 2), with the P2/P1 population spike-amplitude ratio increasing 221% from 0.53 ± 0.29 before stimulation to 1.17 ± 0.09 after stimulation ($p < 0.05$; Student's *t* test; $n = 3$). Recovery of paired-pulse inhibition to baseline levels was not noted, although slight decreases may have occurred in the P2/P1 ratio over a 40-min period of poststimulation observation. Although the baseline P2/P1 ratio in vitro was larger than that in vivo, the results here demonstrate a loss of paired-pulse inhibition in vitro that parallels that observed in vivo. The less-pronounced baseline paired-pulse inhibition in vitro may relate to disruption of inhibitory circuits associated with hippocampal slice preparation.

Loss of paired-pulse inhibition in the dentate gyrus occurs with $GABA_A$ antagonism

To explore a pharmacologic basis for the loss of paired-pulse inhibition with PPS, we recorded field potentials from the granule cell layer in the slice during perfusion of 1–20 μM bicuculline. The P2/P1 population spike ratio increased 221% from a baseline of 0.52 ± 0.16 to 1.15 ± 0.26 ($p < 0.05$; Student's *t* test; $n = 3$), demonstrating that $GABA_A$ inhibition is an important component of paired-pulse inhibition (Fig. 3).

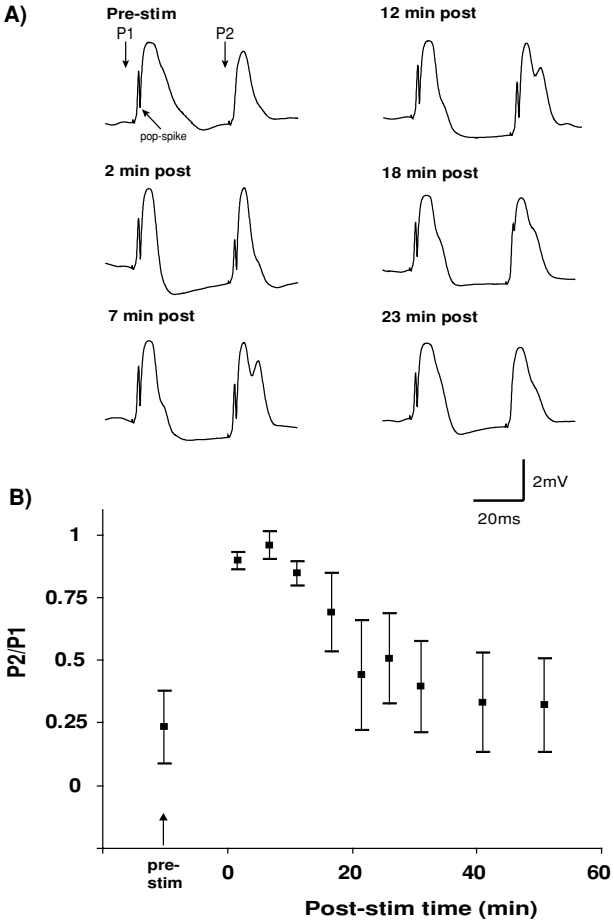

FIG. 1. Loss of paired-pulse inhibition in vivo to brief perforant-path stimulation (PPS) while recording in the dentate gyrus granule cell layer. **A:** Note good paired-pulse inhibition to test pulses before convulsant stimulation, with the presence of a population spike with the first pulse (P1) but not with the second pulse (P2). A prolonged loss of paired-pulse inhibition is noted after 1 min of stimulation (2 Hz continuous with 20 Hz for 10 s, 20 V, 0.1-ms duration) by the emergence of a population spike with the second pulse. Recovery requires nearly 20 min in this example. **B:** Time course of recovery of paired-pulse inhibition after 1 min of PPS. Paired-pulse inhibition was measured by the ratio of population spike amplitudes to stimulation pulses P1 and P2 separated by 40 ms. Boxes are means with error bars as ± SEM (n = 4).

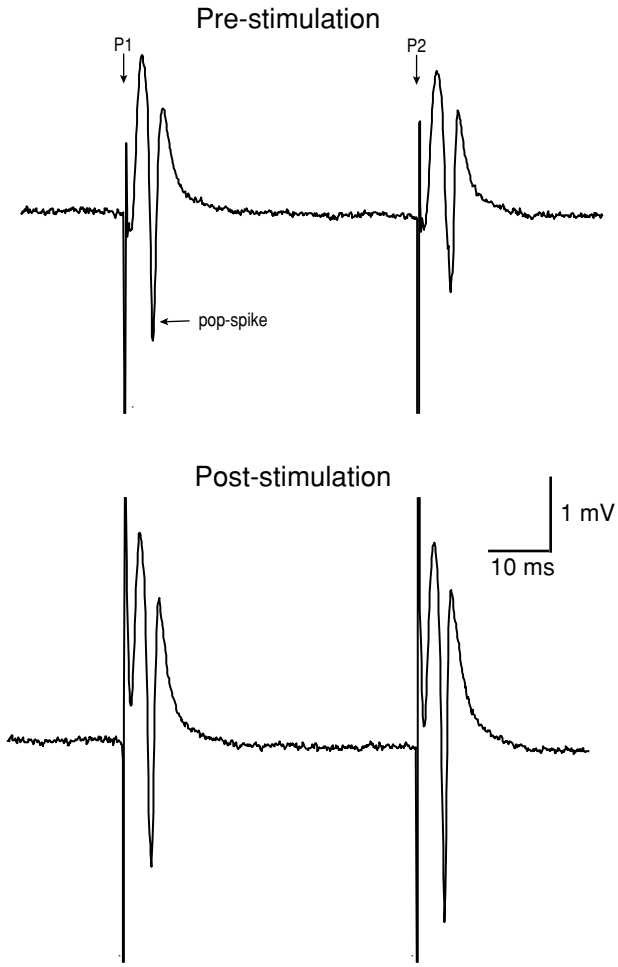

FIG. 2. Decreased paired-pulse inhibition in the hippocampal slice after perforant-path stimulation (PPS; 5 min of 2 Hz continuous with 20 Hz for 10 s/min, 120 V, 0.15-ms duration) while recording field potentials in the dentate granule cell layer.

GABAergic synapses of granule cells may mediate the loss of paired-pulse inhibition seen with brief PPS

Because $GABA_A$ antagonism and PPS can cause loss of paired-pulse inhibition in vitro, we sought to relate effects at $GABA_A$ synaptic receptors more directly to PPS-induced loss of paired-pulse inhibition. We recorded perforant path–evoked IPSCs of granule cells to paired pulses (interstimulus interval, 40 ms) before and after 1–2 min of PPS. The ratio of the peak eIPSC amplitude (P2/P1) decreased 22% (Fig. 4) from 0.96 ± 0.13 before to 0.75 ± 0.10 after PPS ($p < .05$; $n = 3$; paired *t* test), or involved a decrease in amplitude of the single-pulse evoked IPSC (Fig. 5) by 36 ± 11% ($p < 0.05$; $n = 3$; paired *t* test). These findings suggest that effects at GABA synapses, including a reduction of postsynaptic inhibition, may underlie the loss of paired-pulse inhibition measured after PPS by field recordings. Evoked IPSCs are prolonged enough in duration for the residual of the first response to overlap and summate with the second evoked response (Fig. 4), and this may provide a basis for the $GABA_A$-mediated paired-pulse inhibition in field recordings that occurs at short interstimulus intervals (10). The ratio of the eIPSC amplitude of the second compared with first pulse often was <1.0 before PPS, which likely can be attributed to the frequent occurrence of paired-pulse facilitation at baseline in field recordings in vitro compared with in vivo.

DISCUSSION

We find a rapid loss of paired-pulse inhibition to brief PPS in vitro similar to what has been observed in vivo (8). The loss of paired-pulse inhibition can be mimicked by $GABA_A$ antagonism and is predicted by changes in evoked IPSCs at GABA synapses after brief PPS. Our

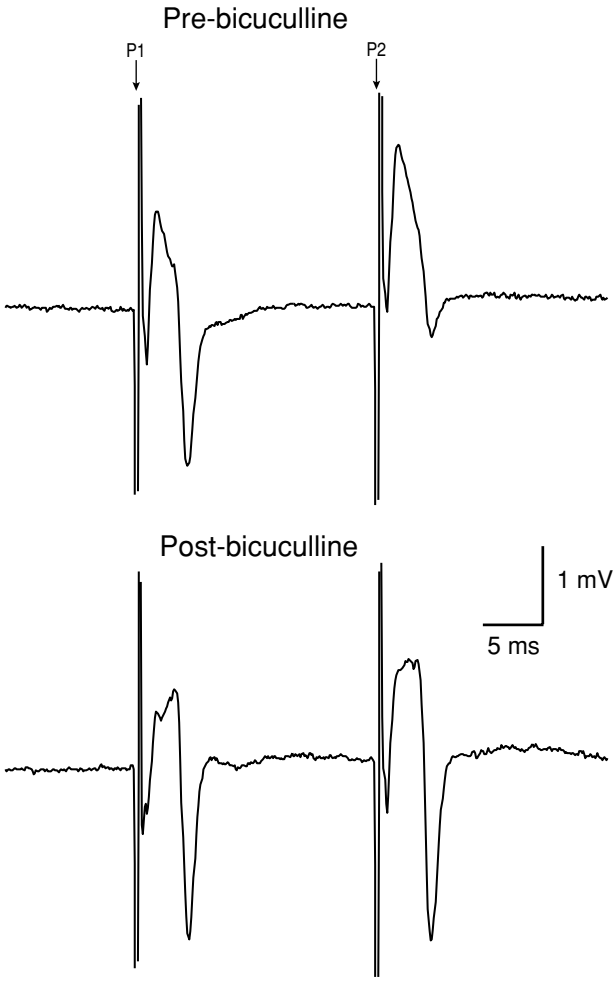

FIG. 3. Loss of paired-pulse inhibition in the hippocampal slice with γ-aminobutyric acid $(GABA)_A$ antagonism (1 μM bicuculline). Field potentials were recorded in the granule cell layer to test pulses of the perforant path fibers before and 10 min after perfusion with bicuculline.

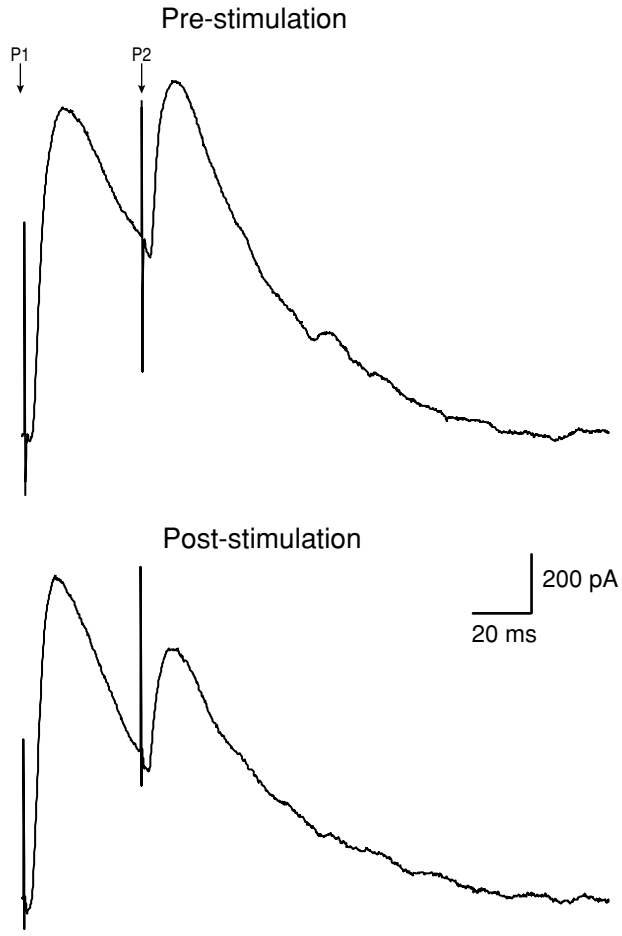

FIG. 4. Paired-pulse evoked inhibitory postsynaptic currents (IPSCs) from whole-cell patch-clamp recordings of dentate granule cells before and after perforant-path stimulation (PPS; 5 min of 2 Hz continuous with 20 Hz for 10 s/min, 90 V, 0.1-ms duration). Before stimulation, note the greater total IPSC for the response to the second test pulse (P2) as compared with the first (P1). After stimulation, the response to the second pulse is diminished relative to the first, consistent with diminished paired-pulse inhibition. Note that residual inhibition from the first pulse contributes to the amplitude from the second. The intracellular electrode solution contained Cs-gluconate with a holding potential of 0 mV.

results suggest that, by 1–2 min of convulsant stimulation of the perforant path, changes in inhibition at GABA synapses correlate with the loss of paired-pulse inhibition of population spikes measured by field recordings.

With the PPS model of SE, interictal spikes occur by 1 min and progress to self-sustaining SE in some rats by 15 min and in nearly all rats after 30 min of stimulation (6). A rapid and progressive loss of potency of GABAergic agents such as BZDs is noted during SE (7), with diazepam (DZP) effectively terminating seizures by 10 min after onset of SE, but failing in all animals by 45 min (3). We have found that postsynaptic $GABA_A$ receptor–mediated inhibition is decreased significantly by 1 h of lithium-pilocarpine SE, largely because of an internalization of postsynaptic $GABA_A$ receptors (13) (which may explain the time-dependent development of the pharmacoresistance to BZDs). The rapid changes to seizure-like stimulation we now note at GABA synapses are consistent with a loss of GABAergic inhibition as an important factor in the initiation phase of SE, whereas glutamatergic activation may be more important with maintenance (9,14).

Among possible mechanisms that can affect GABA synapses within minutes of seizure activity, postsynaptic $GABA_A$-receptor desensitization and receptor internalization (as occurs by 1 h of SE) are considerations. An increase in GABA release and GABA levels occurs during SE (15,16), and micromolar elevations in extracellular [GABA] can lead to $GABA_A$-receptor desensitization (17). We have noted increases in tonic $GABA_A$ currents during SE, consistent with elevated extracellular [GABA], and 5 μM [GABA] levels may cause synaptic $GABA_A$-receptor desensitization adequate for a loss

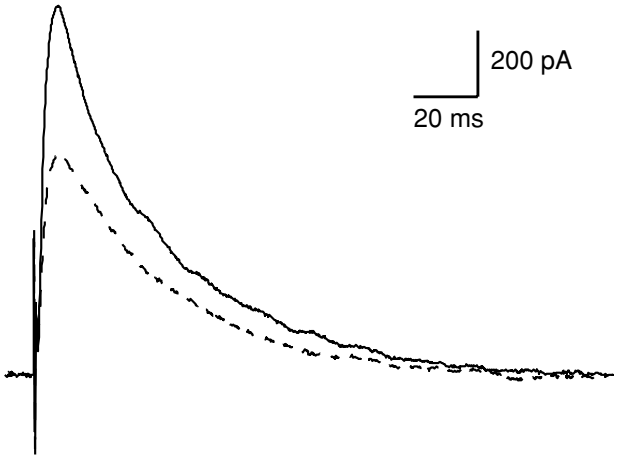

FIG. 5. Evoked inhibitory postsynaptic current (IPSC) responses before (solid) and after (dashed) perforant-path stimulation (PPS; 1 min of 2 Hz continuous with 20 Hz for 10 s, 110 V, 0.1-ms duration) can show a reduction of amplitude with stimulation. The intracellular electrode solution contained Cs-gluconate with a holding potential of 0 mV.

of paired-pulse inhibition (18). Because $GABA_A$ tonic currents parallel the level of cell firing (19), it may be reasonable to attribute increases of GABA release and elevated [GABA] levels (with the potential for postsynaptic receptor desensitization) to increases of neural firing during the onset of SE. Other mechanisms might include activation of signal-transduction pathways involved in $GABA_A$-receptor phosphorylation/dephosphorylation, such as occurs with long-term depression at inhibitory synapses (20), possibly through enhanced calcineurin activity (21) and increased calcium entry from augmented *N*-methyl-D-aspartate (NMDA) activation during SE (22,23).

Many factors can contribute to synaptic depression and paired-pulse inhibition, including presynaptic factors affecting the probability of vesicle release and postsynaptic factors such as receptor desensitization. Increased GABA levels acting at presynaptic sites on inhibitory interneurons have been suggested to cause loss of paired-pulse inhibition in the dentate gyrus (24), and activation of presynaptic $GABA_B$ autoreceptors indirectly can affect postsynaptic $GABA_A$ responses (25–29). However, with a long-lasting effect from PPS on presynaptic $GABA_B$ autoreceptors, the first evoked IPSC would be expected to have a reduced amplitude, similar to what is observed with loss of paired-pulse depression at GABAergic synapses with the $GABA_B$ agonist, baclofen (28,29), or after "priming" or stimulation trains (27). Although a decreased first pulse–evoked IPSC could be observed (Fig. 5), just as often, the first pulse response amplitude was not diminished after PPS (Fig. 4). Furthermore, the short interstimulus intervals we used (40 ms) would minimize pulse-to-pulse effects on presynaptic $GABA_B$ receptors, which are maximal at intervals of 150–200 ms (27,28). At such short interstimulus intervals, it is unlikely that changes in the P2/P1 ratio of paired-pulse responses after PPS would reflect actions at $GABA_B$ autoreceptors. In addition, $GABA_B$ receptors predominantly associate at excitatory glutamatergic synapses (30), and $GABA_B$ activation tends to attenuate the glutamatergic activation of inhibitory neurons (31). Postsynaptic $GABA_A$-receptor desensitization from elevated extracellular GABA levels could explain the loss of paired-pulse inhibition as well as the lack of complete restoration of paired-pulse inhibition with $GABA_B$ antagonism (24). Because our intracellular electrode solution contained cesium, which blocks potassium channels, postsynaptic $GABA_B$ responses were not important in the analysis of our results.

Another factor that potentially could contribute to a loss of paired-pulse inhibition is an increase in intracellular calcium in inhibitory neurons due to the tetanic-like stimulation of PPS, with an increased probability of vesicle release for the first of the paired-pulses (and decreased for the second). Although this may be a contributory factor, such a mechanism would predict a decrease in the population spike for the first pulse after PPS (due to augmented inhibition), which we did not observe. Similarly, after PPS, a decrease in the amplitude of the eIPSC for the first pulse could occur and would not be anticipated by this presynaptic mechanism alone.

Our results suggest that effects at GABAergic synapses occur within minutes of convulsant stimulation, likely involve $GABA_A$ receptors directly, and may explain field potential results in vivo and in vitro, showing early loss of paired-pulse inhibition induced by PPS (8) or seizure activity (32,33). This early alteration of GABAergic inhibition may be an important initial step that eventually progresses to the dramatic loss of synaptic inhibition, postsynaptic $GABA_A$ receptor internalization (13), and pharmacoresistance to BZDs (3–5,34) observed by 1 h of SE.

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