Activity-Dependent Depression of GABAergic IPSCs in Cultured Hippocampal Neurons

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Jensen, Kimmo, John D. C. Lambert, and Morten Skovgaard Jensen. Activity-dependent depression of GABAergic IPSCs in cultured hippocampal neurons. J. Neurophysiol. 82: 42–49, 1999. Short-term depression of monosynaptic GABAergic inhibitory postsynaptic currents (IPSCs) evoked between pairs of cultured rat hippocampal neurons was investigated using dual whole cell patch-clamp recordings. Paired stimuli applied to the GABAergic neuron resulted in paired-pulse depression (PPD) of the second IPSC (IPSC2) at interpulse intervals from 25 to 2,000 ms. CGP 55845A, but not CGP 35348, reduced PPD marginally. Brief paired-pulse applications of exogenous GABA indicated that postsynaptic factors made only minimal contribution to PPD of IPSCs. IPSC1 and PPD was reduced on lowering [Ca2+]o and enhanced on increasing [Ca2+]o. The potassium-channel blocker 4-aminopyridine (4-AP), which increases presynaptic Ca2+ influx, enhanced IPSC1 and PPD. Chelation of residual Ca2+ in the GABAergic boutons with EGTA-AM enhanced PPD. Stimulation of the presynaptic neuron at frequencies (f) ranging from 2.5 to 80 Hz resulted in tetanic depression of IPSCs, which declined rapidly and reached a plateau depending on f and [Ca2+]o. CGP 55845A decreased tetanic depression in the first part of the train, but this could be overcome with continued stimulation. We show that GABAergic IPSCs are robustly depressed by paired-pulse stimulation in cultured hippocampal neurons. The depression of IPSCs is mainly independent of presynaptic GABA receptors and could be caused by depletion of releasable vesicles. Depressed synapses recover with a slow time course, depending on factors that regulate [Ca2+]o in the GABAergic boutons.

METHODS

Hippocampal culture preparation

Pregnant Sprague-Dawley rats were anesthetized by pentobarbital sodium (50 mg/kg ip) at gestational day 17–18. Fetuses were removed and decapitated, and the hippocampi were dissected free. Tissue was triturated mechanically in a HEPES-buffered dissection medium and plated on poly-D-lysine–coated coverslips in 35-mm Petri dishes. Plating medium consisted of minimal essential medium with Earle’s salts and Glutamax-1 (glutamine) supplemented with horse serum (HS, 10%), fetal calf serum (FCS, 10%), penicillin (50 IU ml−1), and streptomycin (50 μg/ml). Cultures were grown in 5% CO2 and 10% O2 at 37°C. Plating medium was fully replaced by 2 ml feeding medium after 1 day in vitro, and thereafter 1 ml was exchanged twice weekly. Feeding medium had the same composition as plating medium except that FCS was omitted and HS was reduced to 5%. The mitosis inhibitors 5′-Fluoro-2′-Deoxyuridine (FUDR, 15 μg/ml) and uridine (35 μg/ml) were added after 3–4 days to inhibit glial overgrowth.

Electrophysiology

Coverslips with the cultured cells were placed in a chamber mounted on an inverted Nikon Diaphot 200 microscope and perfused...
(1 ml/min) with an extracellular (control) medium containing (in mM) 140 NaCl, 3.5 KCl, 2.5 CaCl$_2$, 2.5 MgCl$_2$, 10 glucose, and 10 HEPES; pH 7.35 with NaOH (22 °C), osmolality 305 mosm/kg (Wescor 5500 osmometer). Patch-clamp electrodes (3–6 MΩ) were fabricated from borosilicate glass (1.2 mm OD) on a Flaming/Brown P-97 puller (Sutter Instruments). The presynaptic electrode contained (in mM) 140 KOH, 11 EGTA, 1 CaCl$_2$, 2 MgCl$_2$, 15 NaCl, 10 HEPES, 0.10 leupeptin, 2 MgATP; pH adjusted to 7.3 with methanesulfonic acid, 290 mosm/kg. To increase the driving-force for Cl$^-$ and to block Na$^+$ and K$^+$ currents, the postsynaptic electrode contained (in mM) 120 CsCl, 10 TEACl, 5 QX-314, 11 EGTA, 1 CaCl$_2$, 1 MgSO$_4$, 0.10 leupeptin, 4 MgATP, pH adjusted to 7.3 with CsOH, 290 mosm/kg. Whole cell recordings were made at a holding potential (V$_{holding}$) of ~70 mV using Axopatch 200 and 200A amplifiers in voltage clamp. Excitatory synaptic responses were blocked by 6-cyano-7-nitroquinolin-2-3-dione (CNQX; 10 μM) and dl-2-amino-5-phosphono-pentanoic acid (dl-AP5; 50 μM). GABAergic neurons were identified by the presence of autaptic IPSCs following a 3-ms depolarizing pulse to 0 mV.

Stimulation pulses (3 ms at 0 mV) were delivered by a pulse-generator (Master 8, AMPI) to the presynaptic GABAergic neuron. Paired-pulse stimulation was delivered at interpulse intervals (IPI) ranging from 25 ms to 1 s at a rate of 0.1 Hz and at IPIs of 2–4 s at 0.067 Hz. Stimulus trains consisting of 80 pulses were given at 2.5–80 Hz. Whole cell currents were low-pass filtered at 10 kHz, monitored on a pen recorder (Servogor 220), digitized using an AD converter (Instrutech VR-100 B), and stored simultaneously on a VTR and a computer (Instrutech VR-100 B), and imported into a spreadsheet (Excel v. 7.0a), where statistical calculations were performed. Averaged traces disclosed that the decay of IPSC1 (Fig. 1C) was usually seen. Rundown has previously been calculated to be ~10% after the first 20 min of recording (Jensen et al. 1999b). Statistical calculations were performed. All data are presented as means ± SE with n indicating the number of pairs of neurons tested. Changes were considered to be significant at P values < 0.05.

RESULTS

Monosynaptic GABAergic IPSCs

Monosynaptic GABAergic IPSCs were investigated in pairs of rat hippocampal neurons, which were continuously perfused with CNQX (10 μM) and dl-AP5 (50 μM) to block glutamatergic excitation. The presynaptic GABAergic neuron was stimulated by stepping from ~70 to 0 mV for 3 ms, which evoked a short-latency (1–3 ms) IPSC in most nearby neurons. The IPSC was blocked by bicuculline (10 μM) and had a reversal potential near E$_{Cl}$ ($n = 5$), indicating that it is mediated by GABA$_A$ receptors. In spite of the inclusion of MgATP in the pipette solutions, minor rundown of IPSC amplitudes was usually seen. Rundown has previously been calculated to be ~10% after the first 20 min of recording (Jensen et al. 1999b).

PPD of IPSCs

To study use-dependent depression of the IPSCs, pairs of stimuli at an IPI of 150 ms were delivered at 0.1 Hz to the presynaptic GABAergic neuron. Single traces showed that the amplitude of IPSC$_2$ varied considerably more than the amplitude of IPSC$_1$ (Fig. 1A), and on rare occasions it was larger than IPSC$_2$ (paired-pulse facilitation, not shown). Normally, eight consecutive pairs of IPSCs were averaged and used as the basis for further analysis. Averaged traces disclosed that the amplitude of IPSC$_2$ was always depressed with respect to the first, commonly termed PPD. The time course of PPD was investigated by varying IPI between 25 and 4,000 ms (Fig. 1B). At short IPIs, IPSCs showed temporal summation with IPSC$_2$, riding on the tail of IPSC$_1$. IPSC$_2$ was then measured as the area under the curve and plotted this against PPD (Fig. 2A). Analysis of IPSCs and PPD was correlated to the size of IPSC$_1$ ($P < 0.05$), and for every 100% increase in IPSC$_1$, PPD increased by 33%.

Effect of 4-aminopyridine (4-AP) on IPSCs and PPD

4-AP inhibits A-type K$^+$ channels and delays repolarisation after APs. This prolongs presynaptic Ca$^{2+}$ influx and enhances the probability of transmitter release at cultured hippocampal GABAergic synapses is steeply dependent on Ca$^{2+}$ influx and enhances the probability of transmitter release at cultured hippocampal GABAergic synapses.
transmitter release (Buckle and Haas 1982). During paired stimulation, 4-AP (20–100 μM) enhanced IPSC₁ and PPD (Fig. 2B). From the plot of PPD as a function of IPSC₁ (Fig. 2C: ▲), it is seen that PPD did not increase as steeply with IPSC₂, as was the case in the experiments in which [Ca²⁺]ᵢ was increased. We also wanted to examine recovery from PPD after depletion of the entire readily releasable pool of vesicles. We found that this could be accomplished by perfusing 100 μM 4-AP, which enhanced PPD to nearly 100% at the shortest IPIs. Results from a single pair of neurons are shown in Fig. 2D. The time course of PPD in the presence of 4-AP was similar to the control (t₁/₂ ~500 ms, and full recovery following PPD ~3 s in both cases).

**PPD is reduced by a GABA₄ receptor antagonist**

It has been reported that PPD of IPSCs in cultured hippocampal neurons is completely insensitive to the GABA₄ receptor antagonists 2-OH saclofen (Yoon and Rothman 1991) and CGP 35348 (Wilcox and Dichter 1994). Synaptically released GABA is, therefore, not thought to activate presynaptic GABA₄ receptors in this preparation. Accordingly, we found that bath perfusion of CGP 35348 (100 μM) had no effect on either IPSC₁ or PPD, which was 36.0 ± 6.3% in control compared with 35.1 ± 5.1% in CGP 35348 (P > 0.05, n = 6, paired t-test). The GABA₄ agonist, baclofen (10 μM) depressed IPSCs by 41.8 ± 6.9% (n = 7, not shown), and this effect could be blocked by CGP 35348 (n = 3), demonstrating that this antagonist was indeed able to block actions at presynaptic GABA₄ receptors.

In contrast to CGP 35348, the newer potent blocker CGP 55845A (1–5 μM) (Davies et al. 1993) caused a significant reduction of PPD (Fig. 3B) from 39.4 ± 7.9% to 31.8 ± 10.4%
(P < 0.05, n = 6, paired t-test). Full reversal of the effect on PPD was obtained after wash out of the drug. Because rundown was usually seen, the effect of CGP 55845A on a single IPSC was difficult to evaluate. However, when CGP 55845A was applied through a perfusion pipette that allowed medium exchange within a few seconds (see METHODS), single IPSCs were slightly enhanced (by 7% superimposed on a rundown of 2%, n = 2). This could indicate that presynaptic GABA<sub>B</sub> receptors are tonically activated in control conditions.

IPSCs can be mimicked by exogenous applications of GABA

Investigations of the reversal potentials for pairs of IPSCs showed that E<sub>IPSC</sub> did not change between IPSC<sub>1</sub> and IPSC<sub>2</sub> (n = 2, not shown). To examine whether desensitization of postsynaptic GABA<sub>A</sub> receptors plays a role in PPD, these were activated directly by applying brief (10 ms) pulses of GABA (1 mM) to 25 single neurons using a focal perfusion system (see METHODS). The responses to GABA qualitatively mimicked GABAergic IPSCs, although the response to GABA (Fig. 4Ab) had slower kinetics (t<sub>decay</sub> 82.7 ± 7.3 ms, n = 25) than IPSCs (41.9 ± 5.3 ms). Paired application of GABA was made in 71 trials at different IPIs. The absolute amplitudes of the two responses to GABA were similar, whereas the net amplitude of the second response (GABA<sub>2</sub>) depended on whether a tail of the first response was still present (Fig. 4B). PPD of the net amplitude of GABA<sub>2</sub> was therefore mainly a result of temporal summation. This is as illustrated in Fig. 4Ca, where the decay of GABA<sub>1</sub> (dotted line) is shown in relation to PPD of the GABA responses. The curve for PPD deviates from the decay of GABA<sub>1</sub> in the interval 200–1,000 ms, indicating that there is a slight decrease in postsynaptic responsiveness at IPIs of 200–1,000 ms. Cb: PPD of IPSCs (taken from Fig. 1C) and the decay of IPSC<sub>1</sub> (calculated as above). IPSCs show profound PPD that is independent of the decay of IPSC<sub>1</sub>.

FIG. 3. CGP 55845A reduces PPD. A: IPSCs elicited by paired presynaptic stimulation with an IPI of 150 ms. In control solution, PPD was 39%. CGP 55845A (2 μM) reduced PPD to 34%. The effects of CGP 55845A were superimposed on a small rundown of the responses, which accounts for the apparent incomplete reversal of IPSC<sub>1</sub> on washing. Shown below are IPSCs scaled to the same IPSC<sub>1</sub> amplitude and superimposed to illustrate the reduction of PPD by CGP 55845A. B: CGP 55845A (1–5 μM) reduced PPD significantly from 39.4 to 31.8% (n = 6). PPD was not affected by the rundown of the IPSCs and reversed to 37.6% on washing.

FIG. 4. Responses evoked by exogenous GABA show less paired-pulse depression than IPSCs. Aa: pair of monosynaptic IPSCs evoked by presynaptic stimulation at an IPI of 150 ms displayed PPD of 47%. Ab: GABA<sub>A</sub> receptor–mediated whole cell currents in another neuron elicited by brief applications of 1 mM GABA through a local perfusion system (solid bars; pulse duration 10 ms; IPI 150 ms, average of 4 responses). Responses evoked by exogenous GABA mimicked the IPSCs qualitatively, although they had a slower decay kinetics (t<sub>decay</sub> 39 ms for the 1st GABA<sub>A</sub> response compared with 28 ms for IPSC<sub>1</sub> in Aa). The 2nd GABA<sub>A</sub> response (GABA<sub>2</sub>) reached the same amplitude as the 1st. B: responses from another neuron elicited by applications of GABA at the indicated IPIs. Note that the absolute peak values of the 2 responses were similar, irrespective of IPI. Ca: summary of results from 71 trials in 25 neurons of paired GABA applications at different IPIs. PPD of GABA responses was calculated as the net amplitude of GABA<sub>2</sub> compared with GABA<sub>1</sub> and plotted against IPI (•• as in Fig. 1C). Dotted line (+) represents the decay of GABA<sub>1</sub> calculated from the average t<sub>decay</sub> (82.7 ms) of single GABA responses. This shows that the net PPD of GABA responses is mainly caused by temporal summation, with a slight decrease in postsynaptic responsiveness at IPIs of 200–1,000 ms.Cb: PPD of IPSCs (taken from Fig. 1C) and the decay of IPSC<sub>1</sub> (calculated as above). IPSCs show profound PPD that is independent of the decay of IPSC<sub>1</sub>.
4Ch), indicating that PPD of IPSCs was not affected by temporal summation. Taken together, these data suggest that PPD mainly involves a presynaptic mechanism.

**EGTA-AM increases PPD**

It has recently been reported that the rate of recovery from PPD at excitatory glutamatergic synapses depends on residual Ca\(^{2+}\) in the boutons, which is thought to stimulate refilling of depleted vesicles at the active zones (Dittman and Regehr 1998; Stevens and Wesseling 1998; Wang and Kaczmarek 1998). It has not been established whether a similar mechanism is present at inhibitory GABAergic synapses. To investigate this, we made recordings from presynaptic neurons in the perforated-patch configuration to preserve the internal environment. We evoked pairs of IPSCs at an IPI of 500 ms (each 15 s) and monitored PPD (Fig. 5). After stable control recordings had been obtained, perfusion of the membrane-permeable Ca\(^{2+}\) chelator, EGTA-AM (100 \( \mu \)M), was started. PPD increased over the course the next minute and stabilized at a higher level (Fig. 5B), whereas no effect was seen on IPSC\(_1\). In four pairs of neurons, PPD increased from 26.6 ± 4.1% in control to 42.9 ± 1.7% in EGTA-AM (\( P < 0.05 \), Fig. 5C).

These results indicate that chelation of Ca\(^{2+}\) in the boutons by exogenously applied Ca\(^{2+}\) buffers depresses IPSC\(_2\).

**FIG. 5.** EGTA-AM enhances PPD. A: perforated-patch recording was made from a presynaptic GABAergic neuron, and this was stimulated at an IPI of 500 ms to evoke pairs of IPSCs. PPD was 26% under control conditions. After 5 min extracellular application of EGTA-AM (100 \( \mu \)M), PPD had increased to 38%, whereas there was no effect on IPSC\(_1\). Responses are scaled to the same IPSC\(_1\) and superimposed to the right. B: graph from another pair of neurons showing the effect of perfusing EGTA-AM on PPD measured from single pairs of responses. PPD was on average 21% at the start of the experiment. Two minutes after start of perfusion with EGTA-AM, PPD had increased to a mean value of 48%. C: similar experiments were performed in a total of 4 pairs of neurons. PPD increased from 26.6 ± 4.1 in control to 42.9 ± 1.7% in EGTA-AM.

**Tetanic depression of IPSCs depends on frequency and \([Ca^{2+}]_o\)**

Because a substantial depression of IPSC\(_2\) was observed with paired stimulation, it was expected that IPSCs would be further reduced in response to prolonged train stimulation. We applied train stimuli consisting of 80 pulses at 5, 20, 40, and 80 Hz, respectively. IPSCs showed temporal summation at 20, 40, and 80 Hz, and at 20 and 80 Hz a gradual increase in the plateau current occurred toward the end of the train. Tetanic depression of IPSCs was assessed by measuring the average net amplitude at the end of the train (IPSC\(_{71}\) to IPSC\(_{80}\)), and was 42% at 5 Hz, 67% at 20 Hz, 89% at 40 Hz, and 91% at 80 Hz (vertical scale bars: 5 Hz, 2,000 pA; 20 Hz, 200 pA; 40 and 80 Hz, 1,000 pA).

**FIG. 6.** Tetanic depression of IPSCs. A: single traces from 4 different neurons showing tetanic depression of IPSCs evoked by stimulation with 80 pulses at 5, 20, 40, and 80 Hz, respectively. IPSCs showed temporal summation at 20, 40, and 80 Hz, and at 20 and 80 Hz a gradual increase in the plateau current occurred toward the end of the train. Tetanic depression of IPSCs was assessed by measuring the average net amplitude at the end of the train (IPSC\(_{71}\) to IPSC\(_{80}\)), and was 42% at 5 Hz, 67% at 20 Hz, 89% at 40 Hz, and 91% at 80 Hz (vertical scale bars: 5 Hz, 2,000 pA; 20 Hz, 200 pA; 40 and 80 Hz, 1,000 pA). B: tetanic depression of net IPSCs as a function of stimulus number at the frequencies shown. Thirty-seven trains consisting of 80 pulses were applied to 20 pairs of neurons. For each frequency the net IPSC amplitudes were measured, calculated as a percentage with respect to the 1st IPSC and pooled. Only results from the 1st and last 10 pulses are shown. Net IPSCs reached a relatively stable amplitude after 5–6 pulses, irrespective of stimulus frequency. At 2.5, 10, and 20 Hz IPSCs had increased slightly compared with the plateau earlier in the train, whereas a decrease was seen at 40 and 80 Hz. Results for 5 Hz lay between 2.5 and 10 Hz and are omitted for clarity. C: graph showing tetanic depression of net IPSCs at 40 Hz in different \([Ca^{2+}]_o\). Results are presented as in B and show that tetanic depression increases with \([Ca^{2+}]_o\).
Areas were normalized to the area of a single pretetanic B 7 850 –1,000 ms) period of the train (stippled thin lines in Fig. 6B). Tetanic depression was calculated from the average of the last 10 IPSCs in the train. Tetanic depression was strongly affected by frequency, in contrast to \( \tau_{\text{depr}} \) which did not change systematically with \( f \).

In 4.0 mM Ca\(^{2+}\), the last 10 IPSCs were depressed by 76.3 \( \pm \) 3.1\%, in 2.5 mM Ca\(^{2+}\) by 82.1 \( \pm \) 1.4\% and in 4.0 mM Ca\(^{2+}\) by 89.9 \( \pm \) 1.3\%.

**Effects of CGP 55845A on tetanic depression**

To see whether increasing the level of extracellular GABA could cause a greater activation of GABA\(_B\) autoreceptors, we stimulated the presynaptic neuron at 80 Hz for 1 s and tested the effect of CGP 55845A on the response (Fig. 7, \( n = 8 \)). CGP 55845A reduced tetanic depression during the early part of the train but, surprisingly, not later in the train (Fig. 7). To quantify this differential effect, IPSC areas were measured with respect to the baseline during an early (at 150 –300 ms) and a later (at 850 –1,000 ms) period of the train (stippled thin lines in Fig. 7B). Areas were normalized to the area of a single pretetanic IPSC in each solution. Accordingly, a value of 1.0 means that the IPSC area was the same as the area of a single pretetanic IPSC. In CGP 55845A, the early area was 2.28 \( \pm \) 0.6, which was 16\% larger than in control solution (1.96 \( \pm \) 0.2, \( P < 0.05 \), Fig. 7C). There was no significant difference between the late areas, which were 2.45 \( \pm \) 0.3 in CGP 55845A compared with 2.28 \( \pm \) 0.3 (\( P > 0.05 \)). This indicates that the effect of GABA\(_B\) autoreceptor activation is overcome by continued tetanic stimulation.

**DISCUSSION**

**Presynaptic GABA\(_B\) receptors in cultured neurons**

Activity-dependent depression of IPSPs is a prominent feature of GABAergic synaptic transmission in the mammalian CNS (Thompson et al. 1993). Experiments with hippocampal and cortical brain slices have shown that PPD of IPSPs is partly caused by activation of presynaptic GABA\(_B\) autoreceptors, leading to a reduction in GABA release (Davies et al. 1990; Deisz and Prince 1989; Mott et al. 1993; Nathan and Lambert 1991). In contrast to structurally intact preparations, investigations of PPD in cultured hippocampal neurons have shown that PPD of IPSCs is independent of presynaptic GABA\(_B\) receptor activation (Wilcox and Dichter 1994), despite the fact that functional GABA\(_B\) receptors are present on the presynaptic terminals and can be selectively activated by baclofen (Harri-
that these are located at some distance from the GABA release site. Application of CGP 55845A also caused a small enhancement of single IPSCs. If this reflects tonic activation of the high affinity presynaptic GABA<sub>B</sub> receptors, the concentration of ambient extracellular GABA would probably be in the submicromolar range (Dittman and Regehr 1997).

**Postsynaptic factors in PPD**

Although PPD is probably expressed at a presynaptic locus (Wilcox and Dichter 1994), postsynaptic factors such as a shift in $E_{C1}$ (Thompson and Gähwiler 1989) or desensitization of GABA<sub>A</sub> receptors (Frosch et al. 1992; Oh and Dichter 1992) could contribute to PPD. We can, however, rule out that a shift in $E_{C1}$ contributes to the depression of GABA<sub>A</sub>ergic IPSCs because the reversal potentials for IPSC<sub>1</sub> and IPSC<sub>2</sub> were similar. To investigate whether desensitization is involved in the depression of IPSCs, we made paired applications of exogenous GABA to voltage-clamped neurons in the whole cell mode. The concentration of GABA in the synaptic cleft has been estimated to be $\sim 500$ μM, which saturates the postsynaptic receptors (Maconochie et al. 1994). The brief application of GABA (1 mM) used in our experiments is therefore likely to mimic simultaneous release at all GABAergic synapses on the entire neuron. Our results show that the postsynaptic responsiveness was unchanged following the first application of GABA at all IPIs, thereby illustrating that the receptors were not desensitized after the first pulse. This would indicate that desensitization does not contribute to PPD of the IPSCs.

**Presynaptic mechanism for PPD**

Because activation of presynaptic GABA<sub>B</sub> autoreceptors plays only a minor role in PPD, the most likely mechanism is depletion of releasable vesicles at the active zones. Changing $[Ca^{2+}]_o$ or applying 4-AP are established ways of modulating probability of release at individual release sites by altering presynaptic $Ca^{2+}$ influx (Klapstein and Colmers 1992; Ohno-Shosaku et al. 1994b). The results show that PPD was positively related to the probability of release (Fig. 2C). Because this relationship was investigated at an IPI of 150 ms, PPD asymptoted toward 70%, which is the maximal obtainable PPD (see Fig. 2D). In the presence of 100 μM 4-AP, PPD at the shortest IPIs was close to 100%, and the probability of release was maximized. Although the readily releasable pool was maximally depleted by the first stimulation, the rate of recovery from PPD was nevertheless the same as the control.

Our results indicate that GABAergic boutons are refractory following exocytosis, as has previously been suggested for excitatory synapses in culture (Debanne et al. 1996). Depleted active zones are replenished at a slow rate as found at glutamatergic synapses, where refilling takes several seconds (Dobrunz and Stevens 1997; Stevens and Tsujimoto 1995). Disregarding the marginal contribution from GABA<sub>B</sub> receptors, the plot of PPD as a function of IPI (Fig. 2D) would reflect replenishment of depleted vesicles at boutons that had released on pulse<sub>1</sub>. Furthermore, there is increasing evidence against the involvement of other possible sites in PPD such as axonal branch block (MacKenzie et al. 1996) or inactivation of presynaptic voltage-dependent $Ca^{2+}$ channels (VDCCs) (Dobrunz and Stevens 1997; Mintz et al. 1995). The latter could, however, play a role at IPIs of $<20$ ms (Dobrunz et al. 1997).

**Recovery of synaptic depression**

In three recent reports, it has been shown that recovery from PPD at excitatory glutamatergic synapses is accelerated by residual $Ca^{2+}$ in the boutons (Dittman and Regehr 1998; Stevens and Wesseling 1998; Wang and Kaczmarek 1998). In the rat brain stem, refilling of the readily releasable pool could be slowed by reducing the spike frequency (Wang and Kaczmarek 1998). In the cerebellum, the membrane-permeable $Ca^{2+}$ chelator, EGTA-AM, enhanced PPD and delayed recovery (Dittman and Regehr 1998). Accordingly, biochemical data show that synaptic vesicle dynamics are regulated by internal free $Ca^{2+}$ in the range of 1 μM (reviewed by Burgoyne and Morgan 1995). Our results would indicate that a similar mechanism operates at inhibitory GABAergic synapses (Fig. 5). At a fixed IPI of 500 ms, perfusion of EGTA-AM enhanced PPD from 27 to 43%. This is interesting because GABAergic interneurons are believed to have a strong internal $Ca^{2+}$ buffering capacity (Freund and Buzsáki 1996), which would not allow any significant accumulation of free $Ca^{2+}$ in the boutons following a single action potential. Nevertheless, the results with EGTA-AM suggest that internal $Ca^{2+}$ is indeed elevated after a single stimulus, and that $Ca^{2+}$ might stimulate replenishment of the readily releasable pool. However, the precise mechanism responsible for this presynaptic regulation remains to be determined.

**Tetanic depression of IPSCs**

Given that depletion is mainly responsible for frequency-dependent synaptic depression, IPSCs would decline in response to longer stimulus trains until depletion was in equilibrium with the restorative processes (slow refilling), at which point the IPSCs would stabilize at a plateau level. It is apparent that both the initial decline and the initial plateau level depended on both the stimulating frequency and the probability of release (Fig. 6, B and C). However, although IPSCs were unable to follow the stimulation at the highest frequencies (80 Hz, Fig. 6, A and B), an appreciable GABA<sub>A</sub> current was maintained by asynchronous GABA release that continued 1–2 s after end of tetanization. This component of release has been analyzed in detail elsewhere (Jensen et al. 1999b) and can be blocked by EGTA-AM. It is therefore likely that asynchronous release is caused by an increase in $[Ca^{2+}]_i$ in the boutons during the train stimulation (Jensen et al. 1999b). Bearing this in mind, it was interesting to find that CGP 55845A reduced tetanic depression early in the train, but that this effect declined with continued stimulation (Fig. 7B). GABA<sub>B</sub>-mediated inhibition of N- and P/Q-type VDCCs at the secretory apparatus (Ohno-Shosaku et al. 1994b) was overcome with continued stimulation, probably because transmitter release is maintained during the train by $Ca^{2+}$ entering via L-type VDCCs (Jensen et al. 1999a). Because GABA<sub>B</sub> receptors do not desensitize (Miguel et al. 1995), we have identified an alternative mechanism whereby presynaptic GABA<sub>B</sub> receptor function can be attenuated. This observation is important in the context of understanding the function of GABA<sub>B</sub> autoreceptors during high-frequency activity.

**Conclusion**

We have shown that GABAergic IPSCs in cultured hippocampal neurons show robust PPD that is mediated by a
presynaptic mechanism. We suggest that PPD is caused by depletion of vesicles at the active zones, although spill-over of GABA onto presynaptic GABA$_B$ receptors is also present to a minor degree. Depleted vesicles are replenished at a slow rate that, however, depended on [Ca$^{2+}$]$_i$ in the boutons. One could speculate that the loss of such Ca$^{2+}$-mediated acceleration of vesicular recycling at GABAergic synapses in vivo could lead to an increased susceptibility for epileptic discharges. Finally, the effect of activation of GABA$_B$ autoreceptors is overcome with prolonged presynaptic activity. This observation is important in the context of understanding the actions of GABA$_B$ modulators as antiepileptic agents, and contributes to the understanding of how the action of high extracellular levels of GABA at presynaptic GABA$_B$ receptors is counteracted by accumulation of Ca$^{2+}$ in the boutons during high-frequency activity.

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REFERENCES


