Post-tetanic potentiation of GABAergic IPSCs in cultured rat hippocampal neurones

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1. Dual whole-cell patch-clamp recording was used to investigate post-tetanic potentiation (PTP) of GABAergic IPSCs evoked between pairs of cultured rat hippocampal neurones. Tetanization of the presynaptic neuron at frequencies (f) ranging from 5 to 100 Hz resulted in PTP of the IPSCs. Maximum PTP had a magnitude of 51.6% just after the stimulus train, and lasted up to 1 min. PTP was shown to be dependent on the number of stimuli in the train, but independent of f at frequencies ≥ 5 Hz.

2. Blocking postsynaptic GABA_A receptors with bicuculline during the tetanus did not affect the expression of PTP, showing that it is a presynaptic phenomenon. PTP was strongly affected by changing [Ca^{2+}]_o during the tetanus: PTP was reduced by lowering [Ca^{2+}]_o, and increased by high [Ca^{2+}]_o.

3. PTP was still present after presynaptic injection of BAPTA or EGTA, or following perfusion of the membrane-permeable ester EGTA-tetracetaoxymethyl ester (EGTA AM, 50 μM). On the other hand, EGTA AM blocked spontaneous, asynchronous IPSCs (asIPSCs), which were often associated with tetanic stimulation.

4. Tetanic stimulation in the presence of 4-aminopyridine (4-AP), which promotes presynaptic Ca^{2+} influx, evoked sustained PTP of IPSCs in half of the neurones tested.

5. The results indicate that PTP at inhibitory GABAergic synapses is related to the magnitude of presynaptic Ca^{2+} influx during the tetanic stimulation, leading to an enhanced probability of vesicle release in the post-tetanic period. The increase in [Ca^{2+}]_o occurs despite the presence of high-affinity exogenous and endogenous intracellular Ca^{2+} buffers. That PTP of IPSCs depends on the number, and not the frequency, of spikes in the GABAergic neurone is in accordance with a slow clearing of intracellular Ca^{2+} from the presynaptic terminals.

Activity-dependent changes in synaptic transmission determine the temporal behaviour and output of neuronal networks in the mammalian central nervous system (CNS) (O'Donovan & Rinzel, 1997). At single synapses, different patterns of activity can evoke frequency-dependent facilitation or depression (Dobrunz & Stevens, 1997), which in its simplest form can be studied using paired-pulse protocols in order to evoke paired-pulse facilitation (PPF) or depression (PPD) (McCarren & Alger, 1985). Changes in synaptic strength can also persist for various periods of time following the conditioning stimulation. Post-tetanic potentiation (PTP) lasts for a number of seconds after tetanization (Griffith, 1990), and may be succeeded by short-term potentiation (minutes), long-term potentiation (LTP) or long-term depression (LTD) (hours) (Alger & Teyler, 1976). All the aforementioned types of plasticity have been studied in detail at excitatory glutamatergic synapses in the CNS (Dobrunz & Stevens, 1997). However, at inhibitory GABAergic synapses, only PPD (Davies et al. 1990; Nathan & Lambert, 1991) and PPF (Tanabe & Kaneko, 1996), and LTP and LTD (McLean et al. 1996) have been described. Since PTP of GABAergic IPSPs would be an important factor for the integration of all synaptic activity (and probably for other forms of tetanus-induced synaptic plasticity), it is important to characterize the stimulus patterns required to evoke PTP of IPSCs.

The aim of the present study was, therefore, to demonstrate and characterize PTP at GABAergic hippocampal synapses in vitro. For this purpose, we made paired whole-cell recordings from cultured hippocampal neurones in the presence of glutamate receptor antagonists and stimulated the presynaptic GABAergic neurone to evoke monosynaptic GABA_A receptor-mediated IPSCs. This technique also allowed us to manipulate the internal environment of the GABAergic neurone by altering the composition of the solution in the presynaptic electrode. We observed robust PTP of IPSCs which lasted up to 1 min following tetanic stimulation of the GABAergic neurone. PTP was shown to
depend on the magnitude of presynaptic Ca²⁺ influx and internal Ca²⁺ buffering. Part of this work has been presented in abstract form (Jensen et al. 1998).

METHODS

Hippocampal culture preparation

Pregnant Sprague–Dawley rats were anesthetized by pentobarbital (50 mg kg⁻¹ i.p.) at gestational day 17–18, following which the rats were killed by cutting the major arteries at the heart, in accordance with the guidelines laid down by the Danish Ministry of Justice. Fetuses were removed and decapitated, and the hippocampi were dissected free. The tissue was triturated mechanically in medium containing (mM): NaCl, 137; KCl, 3.5; CaCl₂, 2.5; MgCl₂, 1; KH₂PO₄, 1; HEPES, 10; glucose, 10; and Phenol Red, 0.003; pH 7.3–7.4 with NaOH, and cells were plated on poly-l-lysine-coated coverslips in 35 mm Petri dishes. Plating medium consisted of minimal essential medium with Earle’s salts and Glutamax-1 (Gibco) supplemented with horse serum (HS, 10%), fetal calf serum (FCS, 10%), penicillin (50 i.u. ml⁻¹) and streptomycin (50 μg ml⁻¹). Cultures were grown in 5% CO₂ and 10% O₂ at 37°C (Brewer & Cotman, 1989). 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 mts₁₂ inhibitor 5’-fluoro-2’-deoxyuridine (FUDR, 15 μg ml⁻¹) and uridine (35 μg ml⁻¹) were added after 3–4 days when cultures showed a confluent background.

Electrophysiology

Coverslips with the cultured cells were placed in a stainless steel chamber with a quartz glass bottom mounted on an inverted Nikon Diaphot 200 microscope, and individual neurones were visualized through x200 Normarski optics. The chamber was continuously perfused (1 ml min⁻¹) with an extracellular medium containing (mM): NaCl, 140; KCl, 3.5; CaCl₂, 2.5; MgCl₂, 1; HEPES, 10; glucose, 10; and Heps, 10. pH was adjusted to 7.35 with NaOH (20–22°C). Osmolality was 305 mosmol kg⁻¹ (regularly checked using a WeeCOR 5500 osmometer). Patch-clamp electrodes (tip resistance, 3–6 MΩ) were fabricated from borosilicate glass (o.d. 1.2 mm) on a Flaming–Brown P-97 puller (Sutter Instruments). Excitatory synaptic interactions between neurones were blocked by including 6-cyano-7-nitroquinoline-2,3-dione (CNQX, 10 μM) and DL-2-amino-5-phosphonovaleric acid (AP5, 50 μM) in the perfusion medium. GA/Bergic neurones were initially identified by screening single neurones by whole-cell recording and investigating whether brief stimulation (see below) was followed by an antidromic IPSC. If so, a neurone in close vicinity was then patched and tested for the presence of IPSCs synchronized at a constant latency to the presynaptic stimulation. The presynaptic electrode contained (mM): KOH, 140; EGTA, 11; CaCl₂, 1; MgCl₂, 2; NaCl, 15; Heps, 10; leupeptin, 0.1%; and MgATP, 2; pH adjusted to 7.3 with methanesulfonic acid, 290 mosmol kg⁻¹. The post synaptic electrode solution was designed to increase the driving force for Cl⁻ and to block regenerative Na⁺ and K⁺ currents, and contained (mM): CsCl, 120; TEACl, 10; EGTA, 11; CaCl₂, 1; MgSO₄, 1; leupeptin, 0.1%; MgATP, 4; and QX-314, 2; pH adjusted to 7.3 with CsOH, 290 mosmol kg⁻¹. The perforated-patch configuration, the pipette solution contained (mM): KOH, 140; KCl, 15; Heps, 5; EGTA, 1; and freshly dissolved amphotericin B, 0.32; pH adjusted to 7.3 with methanesulfonic acid, osmolality 290 mosmol kg⁻¹.

Stimulation protocol

Whole-cell recordings were made using Axopatch 200 and 200A amplifiers in voltage-clamp mode at a holding potential (VH) of −70 mV, and stimulation pulse protocols were delivered by a pulse generator (Master 8, AMP). We decided to stimulate the presynaptic neurone in the voltage-clamp configuration for two reasons. Firstly, the presynaptic neurone was clamped at −70 mV between stimuli to prevent spontaneous firing. Secondly, spikes evoked by current-clamp stimulation could have variable latency, resulting in jitter of the IPSCs. The presynaptic neurone was stimulated by stepping from −70 to 0 mV for 3 ms, which evoked a Na⁺-dependent action current in the soma. This was followed by an IPSC in the postsynaptic neurone with a constant latency of between 1 and 3 ms. IPSCs were blocked by tetrodotoxin (300 nM, not shown), indicating that the somatic action current led to breakaway action potentials in the axon. Single control stimuli were given at 0.2 Hz, while stimulus train were delivered at frequencies ranging from 2.5 to 100 Hz. Whole-cell currents were low-pass filtered at 10 kHz, monitored on a pen recorder (Servogor 220), digitized using an A/D converter (Instrutech VR-100 B) and stored simultaneously on a videotape recorder and a Pentium PC equipped with Clampex (pCLAMP version 6.0, Axon Instruments).

Drug application

Active substances were dissolved in water as stock solutions at 1000 times the final concentration, diluted in extracellular medium just before use and perfused through the bath (exchange time, 2–3 min). All changes in [Ca²⁺], were compensated by changes in [Mg²⁺], to keep the total extracellular divalent cation concentration constant at 5 mM. For experiments in which a rapid change between control medium and test solution was required, the neurones were continuously superfused from a 3-barrel gravity-feed pipette (tip opening, ~200 μm). Experiments with perfusion of bicuculline (to block GABAₐ receptor-mediated IPSCs) and solutions with an altered Ca²⁺/Mg²⁺ ratio showed that the local medium was completely exchanged in less than 2 s. Drugs and chemicals were purchased from Sigma except CNQX and n-AP5 (Tocris Cookson). EGTA-tetraacetoxyethyl ester (EGTA AM, Molecular Probes) was prepared as a stock solution dissolved in DMSO. When added to the control solution at the final concentration of 0.1%, DMSO did not affect the IPSCs. Culture media were purchased from Gibco, except for FUDR, uridine and poly-l-lysine (Sigma).

Analysis

IPSC amplitudes were measured on-line using Clampex, whereas offline analysis of amplitudes and areas of IPSCs was performed using Clampfit (pCLAMP). Occasionally, Axotape (Axon Instruments) was used to record traces for visual presentation in figures. All IPSCs evoked by a single stimulation were inspected visually and rejected if spontaneous activity disturbed the measurements. Areas of IPSCs were measured to register both early and late changes in decay kinetics. Post-tetanic potentiation (PTP) is presented as the percentage change in amplitude of a single IPSC evoked shortly (1–4 s) after the stimulus train compared with the mean of ten pre-tetanic IPSCs (peak PTP). PTP was also quantified by measuring the area under the plot of the amplitudes of the first ten post-tetanic IPSCs, which had been normalized to the pre-tetanic IPSCs (PTP area, see inset in Fig. 2A). Post-tetanic asynchronous IPSCs (aIPSCs) were quantified as the area under the trace with respect to the baseline for 1 s following the train (see Fig. 3A). Data were imported into a spreadsheet (Excel version 7.0a) where means and s.e.m.s were calculated, and linear regression and Student’s paired and unpaired t-tests were performed. The
non-parametric Kruskal–Wallis comparison of several groups was made using the statistical software package SOLO (version 6.0.4). All data are presented as means ± s.e.m. with n indicating the number of pairs of neurons tested. Changes were considered to be significant at P values less than 0.05. In some graphs, error bars have been omitted for clarity.

RESULTS

Monosynaptic GABA<sub>A</sub> receptor-mediated IPSCs

Monosynaptic GABA<sub>A</sub> receptor-mediated IPSCs were examined in 82 pairs of hippocampal neurons continuously perfused with CNQX (10 μM) and picrotoxin (50 μM) to block glutamatergic excitation. The presynaptic GABAergic neuron was clamped at −70 mV and stimulated by stepping V<sub>m</sub> from −70 to 0 mV for 3 ms, which probably evoked a break-away action potential (see Methods). This elicited short latency (1–3 ms) IPSCs in most nearby neurons. These responses were identified as GABA<sub>A</sub> receptor-mediated IPSCs, since they were blocked by bath perfusion of bicuculline (10 μM) and had a reversal potential of about +5 mV with a nearly symmetrical Cl<sup>−</sup> gradient across the cell membrane (n = 3). In spite of the inclusion of MgATP in the pipette solutions, minor rundown of IPSC amplitudes occurred. During the first 20 min of recording with low-frequency stimulation, rundown was calculated by linear regression to be 10.1 ± 4.4% (n = 7).

Post-tetanic potentiation of IPSCs

A brief tetanization (80 Hz for 1 s) of the presynaptic GABAergic neuron resulted in potentiation of subsequent single IPSCs elicited at 0.2 Hz (Fig. 1A). This post-tetanic potentiation (PTP) reached a maximum of 51.6 ± 9.2% (n = 19) just after the stimulus train and the duration of the potentiation was about 1 min (Fig. 1C). The rundown of test IPSCs was of no consequence for the expression of PTP, but led to a small underestimation of its amplitude. For analysis of the decay kinetics of single post-tetanic IPSCs, peak amplitudes were scaled to the pre-tetanic control IPSC and the areas measured in order to detect both early and late changes in IPSC decay kinetics. The first four post-tetanic test IPSCs showed significant enhancement in area which ranged from 8.5 ± 3.3% for the first post-tetanic IPSC to 14.4 ± 3.6% for the fourth IPSC (P < 0.05, n = 19). Since these changes in area were often associated with post-tetanic miniature IPSC activity, measurements of amplitudes alone were chosen to quantify the magnitude and time course of PTP.

Stimulus train parameters required to evoke PTP

Since PTP depended strongly on the train parameters (see examples in Fig. 1D), we systematically examined how PTP was related to the frequency of stimulation (f). Trains fixed at 80 stimuli were applied to the presynaptic neurons at frequencies ranging from 2.5 to 80 Hz (Fig. 2A). PTP was quantified for each trial as the area under the curve described by the amplitude of ten single IPSCs following the tetanus (see inset in Fig. 2A). This method of quantification was chosen to reduce variability due to the quantal nature of transmitter release, and to include changes in the later phase of PTP. Results from 67 trials in 50 pairs of neurons disclosed no significant differences between PTP areas obtained at frequencies from 5 to 80 Hz (P > 0.50, Kruskal–Wallis non-parametric comparison of several groups). However, PTP was not observed with presynaptic stimulation at 2.5 Hz (Fig. 2A), showing that the threshold for induction of PTP lies between 2.5 and 5 Hz.

To examine whether PTP is dependent on the number of stimuli, trains consisting of 20–320 pulses given at 5–100 Hz were applied in 116 trials in 54 pairs of neurons. Examples of these results with frequencies of 5, 20 and 40 Hz are shown in Fig. 2B. The slope was calculated by applying linear regression to the plot of PTP areas against the log of the number of stimuli. At all frequencies, PTP increased significantly with the number of stimuli (P < 0.05) and the slopes showed no systematic changes with increasing frequency (5 Hz: slope = 289.1 (24 trials in 17 pairs); 20 Hz, slope = 399.6 (21 trials in 15 pairs); 40 Hz, slope = 351.7 (30 trials in 19 pairs)). All regression lines intercepted the abscissa between 17 and 20 pulses, indicating that the threshold for PTP was the same regardless of the frequency of the train (5 Hz, 18–6 pulses; 20 Hz, 19–2 pulses; 40 Hz, 17–8 pulses). Since PTP depends on the number of stimuli and is independent of f (≥ 5 Hz), a linear regression analysis was performed on the data obtained from experiments with frequencies between 5 and 100 Hz (Fig. 2C). On a semi-logarithmic plot the data could be fitted by a straight line with a slope (329.2) that was significantly different from zero (P < 0.05). Extrapolation showed a threshold for PTP of 18 pulses. Finally, from the results obtained with a high number of stimuli, it seemed that PTP tended to saturate with numbers > 100 (Fig. 2C).

Spontaneous post-tetanic IPSCs are correlated to PTP

In connection with the tetanic stimulation, we often noted an increase in the occurrence of asynchronous IPSCs (asIPSCs) which continued for 1–2 s following the train (Fig. 3A). It has previously been established that similar asynchronous activity recorded at excitatory synapses is caused by accumulation of internal Ca<sup>2+</sup> in the presynaptic terminals (Miledi & Thies, 1971; Cummings et al. 1996). We therefore investigated whether there was a relationship between the magnitude of post-tetanic asIPSCs and PTP. In each cell, the total area of the asIPSCs with respect to the baseline was measured for the first 1000 ms following the train (Fig. 3A). This area was then normalized to the area of a single evoked pre-tetanic IPSC so that the results could be compared between cells. When no asIPSC activity was present, the normalized area included the passive decay back to baseline, which had a typical value of about 0.8 times the area of the single pre-tetanic IPSC. The asIPSC area was on average 5.8 ± 0.9 (n = 29, including neurons in which no asIPSC activity was apparently
There was a correlation between the PTP area (measured as in Fig. 2.A) and the asIPSC area (slope = 45.6, linear regression, $P < 0.001$), indicating that the expression of asIPSCs and PTP are linked to some extent. However, some neurons which displayed a large asIPSC area did not express any appreciable PTP (Fig. 3.B).

The effect of changing $[\text{Ca}^{2+}]_{o}$ on PTP

Next we investigated the effect of manipulating presynaptic $\text{Ca}^{2+}$ influx occurring during the tetanus on the magnitude of PTP. Using local pipette perfusion, the extracellular $\text{Ca}^{2+}$ concentration could be changed in about 1 s (see Methods). Perfusion of the test solution was started immediately after

**Figure 1. Post-tetanic potentiation of IPSCs**

**A,** slow chart recording showing IPSCs elicited by single stimuli at 0.2 Hz. A brief tetanization (80 Hz for 1 s) of the presynaptic neuron was delivered at the filled bar. The first single IPSC following the train showed PTP of 40% compared with the pre-tetanic control IPSC. The potentiation was reversible. **B,** IPSCs from another neuron on a faster time scale. Only the initial 375 ms of the train of IPSCs is shown. The amplitude of the pre-tetanic IPSC is indicated by the arrow on the IPSC recorded 4 s after the train. PTP was 38%. **C,** IPSCs evoked before (Control) and 4 s after the train. The IPSCs have been normalized to the same amplitude and superimposed. The post-tetanic IPSC has a slightly slower decay compared with control. **C,** similar trials with trains of 80 Hz for 1 s were performed in 19 pairs of neurons. Amplitudes were normalized to a control IPSC, which was the mean of ten single responses preceding the train. IPSC amplitude was enhanced by 52% just after the train and decayed back to the control level within 80 s. **D,** to elucidate the stimulation parameters required to evoke PTP, a variety of trains were applied to the presynaptic neuron. Results from four of these protocols are shown here. Stimulation with a 4 s train did not evoke PTP when delivered at 5 Hz (a, 20 pulses, $n=4$) or 10 Hz (b, 40 pulses, $n=3$), but evoked PTP when delivered at 20 Hz (c, 80 pulses, $n=9$) and 80 Hz (d, 320 pulses, $n=4$).
Figure 2. PTP of IPSCs depends on the number of stimuli in the train and not the frequency

A, PTP as a function of frequency (f). Sixty-seven trials were performed in 50 pairs of neurons. Trains were applied at the frequencies shown (log scale) with the number of stimuli held constant at 80. PTP was evaluated by calculating the area under the post-tetanic curve for the first 10 post-tetanic IPSCs (shaded area in inset, subtracting values < 100% when present). For frequencies between 5 and 80 Hz, no differences between the groups were found, the PTP medians being indicated by a cross. Stimulation at 2.5 Hz did not evoke PTP, indicating that the threshold for eliciting PTP is between 2.5 and 5 Hz. B, PTP as a function of the number of pulses. Twenty to 160 pulses were given at 5 (a), 20 (b) and 40 Hz (c) as indicated. For each frequency, PTP was calculated for all trials and plotted against the number of stimuli (log scale). PTP was significantly and positively correlated with the number of stimuli, whereas the slopes obtained by linear regression showed no systematic change with increasing frequency. C, cumulative data from all trials with f ranging from 5 to 100 Hz plotted against number of stimuli. The data were obtained from 116 trials in 54 pairs of neurons. The slope of the regression line was 329.2 (P < 0.05), and it intercepted the abscissa between 18 and 19 pulses. From the few data points with stimuli numbers > 100, it would seem that PTP tends to saturate.
the last pre-tetanic single IPSC, and was switched back to control solution after the stimulus train (Fig. 4A). Perfusion of nominally Ca\(^{2+}\)-free solution ([Mg\(^{2+}\), 5·0 mm] completely blocked IPSCs during the train (40 Hz for 2 s), and abolished PTP (PTP area was 34·4 ± 13·9 in Ca\(^{2+}\)-free solution, n = 3, P > 0·05 when tested against zero, not shown). On varying [Ca\(^{2+}\)]\(_o\) between 1·2 and 4·0 mm, we found that the amplitude of the first IPSC after the train (evoked in 2·5 mm Ca\(^{2+}\)) was positively correlated to [Ca\(^{2+}\)]\(_o\) during the train. PTP of the first post-tetanic IPSC following 1·2 mm Ca\(^{2+}\) was −2·4 ± 5·9 % (P < 0·05, n = 6) (which increased to 29·1 ± 13 % for the second IPSC), 42·6 ± 16 % in the continuous presence of 2·5 mm Ca\(^{2+}\), and 63·6 ± 21 % following 4·0 mm Ca\(^{2+}\) (P < 0·05, n = 5). PTP areas (measured as in Fig. 2A) also increased with [Ca\(^{2+}\)]\(_o\) and were 163 ± 46 following 1·2 mm Ca\(^{2+}\), 278 ± 56 during 2·5 mm Ca\(^{2+}\), and 337 ± 90 following 4·0 mm Ca\(^{2+}\) (Fig. 4B).

PTP does not depend on activation of postsynaptic GABA\(_A\) receptors

To test whether PTP is dependent on activation of postsynaptic GABA\(_A\) receptors, bicuculline (10 μm) was applied from the local perfusion pipette for the duration of the stimulation train (Fig. 4C) (n = 2). While bicuculline completely abolished postsynaptic responses during the train (Fig. 4C), PTP was not different from that evoked in control solution. This indicates that PTP occurs at a presynaptic locus.

The effect of presynaptic intracellular BAPTA on PTP

Although EGTA and BAPTA have similar affinities for Ca\(^{2+}\), the on-rate for chelation by BAPTA (10\(^8\) M\(^{-1}\) s\(^{-1}\)) is two orders of magnitude faster than for EGTA (10\(^6\) M\(^{-1}\) s\(^{-1}\)) (Deisseroth et al. 1996). It has been proposed that calcium ions associate with the secretory machinery at a rate

![Figure 3. Relationship between PTP and asynchronous post-tetanic IPSCs](image)

**Figure 3. Relationship between PTP and asynchronous post-tetanic IPSCs**

_A_, trace from a neuron which showed asIPSCs during and following a train of 80 Hz for 1 s. The asIPSC activity was quantified as the area under the trace for 1 s following stimulation with respect to the baseline. This area (dashed line) was normalized to the area of the single pre-tetanic evoked IPSC (shown to the left) and was in this case 9·2. This neuron showed PTP of 82%. **B**, scatter plot showing the relationship between PTP of IPSCs and the asIPSC area, measured as in A (n = 29). Trains were delivered at 80 Hz for 1 s, and the PTP area was measured as in Fig. 2A. PTP increased with the asIPSC area (linear regression, P < 0·001). Note that a number of neurones displayed a substantial asIPSC activity, but little PTP, though the reverse was not seen.
Figure 4. Changing $[\text{Ca}^2+]_o$ during the train modulates PTP

A, representative traces showing the effect of changing $[\text{Ca}^2+]_o$ during tetanic stimulation. The middle record shows control responses in 2.5 mM Ca$^{2+}$ throughout the recording. Solutions containing 1.2 mM Ca$^{2+}$ (upper record) and 4.0 mM Ca$^{2+}$ (lower record) were applied 1–2 s before a tetanus at 40 Hz for 2 s was delivered, and application was terminated shortly afterwards. Pre- and post-tetanic single IPSCs evoked in 2.5 mM Ca$^{2+}$ are shown on either side of the trains. During the train, IPSCs showed tetanic depression which was reduced in 1.2 mM Ca$^{2+}$ and enhanced in 4.0 mM Ca$^{2+}$. PTP of the IPSCs showed a similar dependence on the level of extracellular Ca$^{2+}$ present during the train. This is illustrated to the right, where the post-tetanic responses elicited after returning to 2.5 mM Ca$^{2+}$ are superimposed (amplified and displaced for clarity). B, graph showing PTP area as a function of $[\text{Ca}^2+]_o$ perfused during the train. PTP was calculated as shown in the inset in Fig. 2A. PTP was absent when a nominally Ca$^{2+}$-free solution was perfused during the train, and increased with increasing $[\text{Ca}^2+]_o$. Data were obtained from 22 trials in 10 pairs of neurones. Individual $n$ values are indicated. C, representative traces showing PTP evoked in control solution by a train of 80 Hz for 1 s (upper trace), and with bicuculline (10 μM) present for the duration of the train (lower trace). The presence of bicuculline during the train completely blocked the postsynaptic response and asynchronous activity, but did not affect the induction of PTP.
intermediate between that of chelation by EGTA and BAPTA (Heidelberg et al. 1994). This leads to a depression of IPSCs by BAPTA (Spigelman et al. 1996).

One mechanism for PTP could be that calcium ions bind to a trigger with fast forward kinetics. To test the ability of BAPTA to affect PTP, we injected BAPTA via the presynaptic electrode. A presynaptic electrode solution containing 11 mM BAPTA, instead of 11 mM EGTA, was used in five experiments (Fig. 5). Four to six minutes after establishing whole-cell recordings from the presynaptic neurones, IPSCs were depressed by $26.1 \pm 6.6\%$ compared with just after membrane rupture. In the presence of BAPTA, absolute IPSC amplitudes progressively increased during train stimulation at 80 Hz for 1 s (tetanic facilitation, Fig 5A). BAPTA did not block asIPSC activity associated with the stimulus train. Peak PTP was dramatically enhanced with BAPTA (241 \pm 87\%, $P < 0.05$, unpaired t test) compared with EGTA-containing electrodes (51.6 \pm 9.2\%, Fig. 5B). PTP area was 661 \pm 277 with BAPTA which is nearly three times greater than with EGTA (224 \pm 38). These data show that the relative PTP increases when the pre-tetanic probability of release is lowered by using a more rapid Ca$^{2+}$ chelator, and that the PTP process probably does not depend on a Ca$^{2+}$ sensor with a fast on-rate for Ca$^{2+}$.

**The effect of EGTA AM on asIPSCs and PTP**

Activity-dependent release of synaptic vesicles relies on the influx of Ca$^{2+}$ through voltage-dependent Ca$^{2+}$ channels (VDCCs), and is enhanced when the ambient level of free Ca$^{2+}$ within the terminal is elevated (Heidelberg et al. 1994). To gain insight into the Ca$^{2+}$-dependent mechanisms underlying asIPSCs and PTP, we manipulated the presynaptic Ca$^{2+}$-buffering capacity using EGTA. Firstly, the effect of endogenous buffers was investigated by making recordings in the perforated-patch mode whilst delivering trains of 80 Hz for 1 s to the presynaptic GABAergic neuron ($n = 3$). In these experiments, asIPSCs were observed during and following the train (Fig. 6A), while peak PTP was $50.9 \pm 6.0\%$. In another population of neurones, whole-cell recordings were made from the

*Figure 5. Presynaptic injection of BAPTA does not block PTP*

A, representative traces showing the effect of using BAPTA (11 mM) instead of EGTA (11 mM) in the presynaptic intracellular solution. During the presynaptic tetanus of 80 Hz for 1 s, the summed IPSCs progressively increased in amplitude during the first part of the train, which contrasts with the tetanic depression seen with EGTA-containing electrodes (e.g. Fig. 4A and C). Tetanic stimulation was followed by substantial spontaneous activity, which lasted for about 1 s. The first post-tetanic IPSC (right) was enhanced by 567\% compared with the pre-tetanic control. B, peak PTP with BAPTA ($\blacklozenge$) was 341\%, which was significantly larger (*$P < 0.05$, $n = 5$) than for EGTA-filled neurones ($\blacktriangle$, $n = 19$).
pre-synaptic GABAergic neurone using electrodes containing 11 mM EGTA (control solution). These neurones showed PTP of 58.8 ± 11%, and asIPSC activity of a similar magnitude to that seen with perforated-patch recordings (n = 29, Fig. 6A). In five of the whole-cell recordings, the cell-permeable ester EGTA AM (50 μM) was perfused after recording control responses. EGTA AM enters the terminals by uptake and ‘trapping’, and EGTA accumulates intra-

Figure 6. EGTA AM inhibits asIPSCs, but not PTP of IPSCs
A, representative traces from three experiments reflecting different levels of Ca²⁺ buffering in the pre-synaptic GABAergic neurone. Aa, recording in the perforated-patch mode, so as not to introduce any exogenous Ca²⁺ chelators. Stimulation was accompanied and followed by asIPSC activity and PTP of the evoked IPSC. Ab, whole-cell recording using an intracellular solution containing 11 mM EGTA gave similar results to the perforated-patch recording. Ac, as in b but with additional extracellular perfusion with 50 μM EGTA AM for 10 min. This abolished the asIPSCs, but did not block PTP of the evoked IPSC. Trains were elicited at 80 Hz for 1 s. B, histogram summarizing the effect of the three different treatments shown in A on the asIPSC area measured as in Fig. 3A. EGTA AM significantly depressed the asIPSC area (⁎P < 0.05) compared with the perforated-patch (n = 3) and whole-cell recordings (n = 29). C, graph showing that EGTA AM (50 μM) had no effect on whole-cell recordings of PTP. Tetanic stimulation at 80 Hz for 1 s was delivered to the pre-synaptic neurones in control solution (▲) and again after 10 min of extracellular perfusion of 50 μM EGTA AM (●). In contrast to the profound effect on asIPSCs, EGTA AM had no effect on either the amplitude or the time course of PTP of IPSCs (P > 0.05, n = 5, error bars have been omitted for clarity).
cellularly following cleavage by unspecific esterases (Cummings et al. 1996). Ten minutes after the start of perfusion of EGTA AM, the aSIISC area was significantly smaller ($P < 0.05$, unpaired $t$ test, Fig. 6A and B), and was only marginally greater than the ratio of 0.8 when no aSIISC activity was present. This shows that the rise in [Ca$^{2+}$], after stimulation at 80 Hz for 1 s did not exceed the threshold for asynchronous vesicle release in the presence of EGTA AM. The differential effect of EGTA (11 mM) injected via the presynaptic neurone, and of perfusion of EGTA AM (50 μM), on aSIISCs (not blocking and blocking aSIISCs, respectively), indicates that the boutons only attained a low concentration of EGTA using presynaptic injection.

EGTA AM had no effect on PTP (Fig. 6C). Ten minutes after addition of EGTA AM, PTP had increased slightly (Fig. 6C). PTP amplitude increased from $76.2 \pm 43.7$ to $84.4 \pm 38.4\%$ ($P > 0.05$), while PTP area increased from $244 \pm 97.2$ to $281 \pm 123$ ($P > 0.05$). Prolonged exposure (for up to 30 min) of the neurones to EGTA AM did not induce further changes in PTP.

The effect of 4-AP on PTP of IPSCs

4-AP blocks the A-type K$^+$ channel, which reduces repolarization following the action potential and will increase presynaptic influx of Ca$^{2+}$ during tetanic stimulation. The effect of 4-AP on PTP was tested in seven experiments. In each pair of neurones, PTP was elicited in control solution and subsequently in the presence of 4-AP.

**Figure 7.** Sustained PTP of IPSCs induced by tetanization in the presence of 4-AP

*4-A* represents traces showing the effect of 4-AP on PTP. Single IPSCs were elicited at 0.2 Hz in the presence of 4-AP (20 μM). Post-tetanic responses are shown for 20, 40 and 40 min, where the potentiation was 64, 40 and 34%, respectively. Recording was continued for 55 min, at which point the IPSC was still potentiated by 21%. B, sustained PTP as shown in A was observed in 3 out of 7 pairs of neurones tested after tetanization in 4-AP (20–50 μM). Results pooled from these three pairs are shown in the graph. Trains of 80 Hz for 1 s were applied in control solution (○) and subsequently in the presence of 4-AP (20–50 μM) (●). In 4-AP, baseline IPSC amplitudes were enhanced by 14.3% (to the dashed line). Immediately after tetanization, peak PTP in 4-AP was 70% (with respect to the pre-tetanic level), compared with 53% for the control. In 4-AP, however, IPSCs showed sustained potentiation (potentiation after 1 min was 43%; after 4 min it was 39%, as averaged from four consecutive responses). The two curves were significantly different at both 1 and 4 min ($P < 0.05$).
effects of 4-AP were reversible on washing. To pre-stimulate levels over the course of 1 min and the effects of 4-AP were reversible on washing.

**DISCUSSION**

**Post-tetanic potentiation at GABAergic synapses**

This report represents the first characterization of PTP at inhibitory GABAergic synapses in the hippocampus. In previous studies which focused on LTP of isolated IPSPs, sampling frequencies were generally too low to detect the presence of PTP (Xie et al. 1995; Komatsu, 1996) or PTP was superimposed upon different degrees of sustained potentiation of the IPSCs (Glaum & Brooks, 1996). Qualitatively similar PTP has been demonstrated at cholinergic inhibitory synapses in invertebrate neurons (Gardner, 1986), where PTP was 80% and lasted about 2 min.

**Mechanism of PTP**

PTP at the neuromuscular junction was originally ascribed to the accumulation of free intracellular Ca^{2+} in the terminals as a consequence of tetanic activity (residual Ca^{2+}; Katz & Miledi, 1968; Rosenthal, 1969). This explanation for PTP has subsequently been substantiated at the crayfish neuromuscular junction by the use of Ca^{2+} imaging (Delaney & Tank, 1994), application of modulators of intracellular Ca^{2+} stores (Tang & Zucker, 1997) and flash photolysis of Ca^{2+} chelators (Kamiya & Zucker, 1994), and at hippocampal excitatory synapses using Ca^{2+} imaging (Reggehr et al. 1994). During the tetanus, Ca^{2+} will enter through VDCCs and bind to the Ca^{2+} sensor at the secretory apparatus, as well as to other mobile and immobile Ca^{2+} buffers in the terminal (Neher, 1998). Despite this buffering, [Ca^{2+}], is raised following the tetanus (Cummings et al. 1996), which increases the probability of vesicle release.

In our experiments, PTP was still present when the postsynaptic GABA_A receptors were blocked during the stimulus train, indicating that PTP is a presynaptic phenomenon (Fig. 4C). PTP was positively correlated to the amount of extracellular Ca^{2+} present during the stimulus train, which would be in accordance with a Ca^{2+}-dependent increase in the probability of vesicle release during the post-tetanic period. PTP increased when the basal probability of release was lowered by presynaptic injection of BAPTA. The action of BAPTA on the probability of release is likely to be caused by a direct effect on the secretory apparatus (see Results). Inhibition of action potential conduction is unlikely to be involved, since millimolar concentrations of the BAPTA derivatives fur-2 and fluo-3 do not cause distal block of action potential propagation in cultured cortical or hippocampal neurons (MacKenzie et al. 1996; MacKenzie & Murphy, 1998).

PTP was positively correlated to the magnitude of post-tetanic asynchronous release (Fig. 3), again underlining the association between Ca^{2+} accumulation and the PTP process. Nevertheless, the occurrence of aIPSCs and PTP were not inextricably linked. Thus, in one-third of the neurons showing marked aIPSC activity, only minimal PTP was induced (Fig. 3). Whether this reflects differences in the Ca^{2+} affinities of the sensors for PTP and aIPSCs in subsets of GABAergic neurons, or is due to a difference in the Ca^{2+}-buffering/clearing mechanisms, cannot be determined from the present results.

**Internal Ca^{2+} buffering**

Asynchronous transmitter release is blocked by EGTA AM at both excitatory (Cummings et al. 1996) and inhibitory synapses (Rumpel & Behrend, 1999) and therefore probably requires diffusion of Ca^{2+} through the cytosol. This contrasts with synchronous release which is usually unaffected by EGTA AM, partly because the Ca^{2+} channels are thought to be in close association with the docked vesicles (Sheng et al. 1996; Mochida et al. 1996), and the binding rate of Ca^{2+} by EGTA is much slower than that by the vesicle-associated Ca^{2+} sensor. However, in giant calyx-type synapses in the rat brainstem (Forasythe, 1994), high concentrations of EGTA injected directly into the presynaptic terminal are able to block asynchronous release (Borst & Sakmann, 1996), suggesting that Ca^{2+} entry through a number of dispersed calcium channels is needed to release a vesicle at this particular synapse.

In the present report, we show that asynchronous release is not affected by a relatively high concentration (11 mM) of either EGTA or BAPTA in the presynaptic electrode. While it is likely that BAPTA and EGTA diffuse right out to the terminals, the concentration is probably much lower than in the presynaptic pipette. For a given concentration of EGTA and BAPTA, the total Ca^{2+}-buffering capacity is similar (Spigelman et al. 1996). Since asynchronous release depends on the ambient level of intracellular Ca^{2+}, it would not be expected to be differentially affected by EGTA or BAPTA (compare Figs 5A and 6A). When using EGTA AM, EGTA would be concentrated in the neuron and probably reach a much higher level in the terminals than can be attained using the patch electrode. This would further limit the increase in [Ca^{2+}] and diffusion of Ca^{2+} through the cytosol, and block asynchronous vesicle release. On the other hand, PTP was not blocked by EGTA AM, indicating that Ca^{2+} chelation by EGTA was insufficient to prevent activation of the sensor. PTP was actually enhanced by presynaptic injection of BAPTA. This is mainly because BAPTA reduces
the basal release and therefore the size of the pre-tetanic IPSC. Moreover, the Ca\(^{2+}\) that enters during the stimulus train will bind to BAPTA and less will be available to buffer the transient rise in [Ca\(^{2+}\)], evoked by a single stimulation during the post-tetanic period. Synaptotagmin I is thought to be the sensor and trigger for vesicle release, and binding of four calcium ions is probably required to fully activate the release process (Geppert et al. 1994). Binding of Ca\(^{2+}\) to the first site (which has a suggested dissociation constant of 100 nM), would prime the synaptotagmin I–vesicle complex which may be the mechanism underlying PTP (Bertram et al. 1996), whereas asynchronous release probably requires substantially higher Ca\(^{2+}\) concentrations (Heidelberger et al. 1994).

**Induction of PTP**

Ca\(^{2+}\) entry associated with 20 presynaptic spikes was the threshold for evoking PTP. Above this threshold, PTP increased with every extra spike up to a maximum of around 100 spikes. PTP could not be induced when the GABAergic neurone was stimulated at 2.5 Hz, but developed fully when the frequency was increased to 5 Hz. This shows that the spatial and temporal summation of [Ca\(^{2+}\)], was not sufficient for generation of PTP at interpulse intervals greater than 200 ms. The Ca\(^{2+}\)-clearing mechanisms therefore require about 300 ms to keep the level of intracellular Ca\(^{2+}\) below the threshold for PTP. Calcium ions are cleared from the cytoplasm by Na\(^{+}\)-Ca\(^{2+}\) exchange (Reuter & Porzig, 1995) and active extrusion by Ca\(^{2+}\)-dependent ATPase. It has recently been suggested that the low-affinity/high-capacity Na\(^{+}\)-Ca\(^{2+}\) exchange mediates the initial rapid clearing of Ca\(^{2+}\), while the high-affinity/low-capacity plasma membrane Ca\(^{2+}\)-ATPase ensures that [Ca\(^{2+}\)] in the terminals is ultimately restored to its resting level (Regehr, 1997). If significant amounts of Na\(^{+}\) accumulate intracellularly as a result of spiking and exchange with Ca\(^{2+}\), activity of the Na\(^{+}\)-Ca\(^{2+}\) exchanger is depressed (or even reversed), and clearing of Ca\(^{2+}\) is delayed (Bouron & Reuter, 1996). Ca\(^{2+}\) clearing would also be prolonged if Ca\(^{2+}\) loading of mitochondria occurred during the tetanus (Tang & Zucker, 1997).

We found that PTP at cultured GABAergic synapses (recorded at 20–22°C) is shorter in duration than at hippocampal excitatory synapses (5 min at 22°C; Tang et al. 1996) or at the neuromuscular junction (5–10 min at 20°C; Rosenthal, 1969). This could indicate that GABAergic boutons have an intrinsically stronger Ca\(^{2+}\)-buffering/clearing system. Various Ca\(^{2+}\)-binding proteins, including parvalbumin and calciretin, are selectively located in some GABAergic neurones (Freund & Buzsáki, 1996), and may play an important role in buffering free [Ca\(^{2+}\)] in GABAergic terminals.

**Sustained potentiation of IPSCs**

Long-term plasticity of GABAergic responses (Xie et al. 1995) has recently been termed LTD\(_{GABA}\_) and LTP\(_{GABA}\_) (McLean et al. 1996). In about half the neurones studied here, tetanic stimulation in the presence of 4-AP resulted in a sustained potentiation of the IPSC, which persisted after washout of 4-AP. 4-AP enhances presynaptic Ca\(^{2+}\) influx, and sustained potentiation of IPSCs may require a specific level of intracellular Ca\(^{2+}\) to be achieved during stimulation, as has been suggested for excitatory glutamatergic synapses (Stanton, 1996). While it has been suggested that sustained potentiation of GABAergic IPSCs occurs at a presynaptic locus (Glauum & Brooks, 1996), it has also been shown that LTP\(_{GABA}\_) requires postsynaptic Ca\(^{2+}\) influx, which is triggered by GABA\(_A\) receptor-mediated depolarization (McLean et al. 1996). In our cultured hippocampal neurones, a significant postsynaptic change in [Ca\(^{2+}\)], is unlikely to occur during GABA\(_A\) receptor-mediated activity, since the boutons are located close to the soma (Benson & Cohen, 1996; K. Jensen, unpublished observation), which was clamped at a hyperpolarized membrane potential with an electrode containing EGTA. We therefore consider it most likely that presynaptic mechanisms are involved in the expression of sustained potentiation of IPSCs, and that subtle grading of the Ca\(^{2+}\) levels in the GABAergic boutons could switch the transient potentiation, which characterizes PTP, into a sustained potentiation.

**Functional consequences**

PTP of IPSPs will participate in the integration of synaptic inputs, and modulate postsynaptic behaviour. PTP of IPSPs would also participate in synaptic plasticity at excitatory synapses such as LTP and LTD, which depend strongly on the frequency and pattern of stimulation (Larson et al. 1986; Bear & Malenka, 1994; Stanton, 1996). Accordingly, activation of GABAergic interneurones at even relatively low frequencies (>5 Hz) will evoke PTP of IPSPs. Finally, PTP of IPSPs could be important in pathophysiological states such as epileptic activity, where high-frequency firing in GABAergic interneurones during ictal activity would evoke PTP of the IPSPs, thereby facilitating cessation of the seizure activity.

**Conclusions**

We have examined the consequences of tetanus-induced Ca\(^{2+}\) influx in presynaptic GABAergic boutons in hippocampal neurones. The rise in [Ca\(^{2+}\)], probably peaks just after the tetanic stimulation, where substantial asynchronous GABA release occurred. During the following minute, the GABAergic IPSCs displayed PTP which resulted from an increase in the probability of release in response to a single stimulation. For PTP to develop, the GABAergic neurones must be activated at least 20 times at 5 Hz or above. PTP of IPSCs could be converted into a sustained potentiation when transient K\(^+\) channels were blocked by 4-AP, which would increase stimulus-induced Ca\(^{2+}\) entry into the terminals. This suggests that the level of presynaptic Ca\(^{2+}\) entry may determine whether the synaptic enhancement is transient or sustained.


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