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Ca^{2+} Buffer Saturation Underlies Paired Pulse Facilitation in Calbindin-D28k-Containing Terminals

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Summary

Ca^{2+} buffer saturation was proposed as a mechanism of paired pulse facilitation (PPF). However, whether it operates under native conditions remained unclear. Here we show that saturation of the endogenous fast Ca^{2+} buffer calbindin-D28k (CB) plays a major role in PPF at CB-containing synapses. Paired recordings from synaptically connected interneurons and pyramidal neurons in the mouse neocortex revealed that dialysis increased the amplitude of the first response and decreased PPF. Loading the presynaptic terminals with BAPTA or CB rescued the effect of the CB washout. We extended the study to the CB-positive facilitating excitatory mossy fiber-CA3 pyramidal cell synapse. The effects of different extracellular Ca^{2+} concentrations and of EGTA indicated that PPF in CB-containing terminals depended on Ca^{2+} influx rather than on the initial release probability. Experiments in CB knockout mice confirmed that buffer saturation is a novel basic presynaptic mechanism for activity-dependent control of synaptic gain.

Introduction

Short-term modifications in the efficacy of synapses occur in synaptic connections of the central nervous system when the presynaptic neuron is repetitively active. Successive unitary postsynaptic potentials (PSPs), evoked by a train of presynaptic action potentials (APs), can increase (facilitate) or decrease (depress) in amplitude, depending on the identity of the two neurons that form a connection (Reyes et al., 1998; Thomson, 1997; Thomson and Deuchars, 1997; Thomson et al., 1993). An increase of the probability of transmitter release caused by successive APs in the presynaptic terminal is postulated to underlie short-term facilitation of PSPs (Atluri and Regehr, 1996; Katz and Miledi, 1968; Rahamimoff, 1968; Rozov et al., 2001; Winslow et al., 1994; Zucker, 1989, 1996). Short-term synaptic enhancement occurring in the time scale of tens of milliseconds to several seconds is thought to be a calcium-dependent process (Fisher et al., 1997). The intracellular concentration of calcium at the release site depends on the distance between Ca^{2+} channels and the Ca^{2+} sensor as well as on the properties of endogenous Ca^{2+} buffers (Neher, 1998).

Three different models have been proposed to explain the relationship between Ca^{2+} concentration in the terminal and paired pulse facilitation (PPF). Katz and Miledi (1968) proposed the residual calcium hypothesis. According to this hypothesis, facilitation is caused by the increased level of residual Ca^{2+} remaining in the terminal after each AP, which adds to the Ca^{2+} influx resulting from subsequent APs, increasing the probability of transmitter release. In another model it has been suggested that facilitation involves an additional high-affinity Ca^{2+} binding site with slow unbinding kinetics, which operates cooperatively with the "main release sensor" (Atluri and Regehr, 1996). The third hypothesis proposes that facilitation can occur as a result of progressive and local saturation of fast endogenous Ca^{2+} buffers in the terminal during a train of APs, thus resulting in a gradual increase of the Ca^{2+} concentration at the release site (Rozov et al., 2001). The partial buffer saturation hypothesis implies that fast Ca^{2+} buffers can effectively bind Ca^{2+} competing with the Ca^{2+} sensor during the first AP. By the time of the second AP, a fraction of the buffer remains bound to Ca^{2+}, allowing more Ca^{2+} to reach the sensor, resulting in increased release probability. In a previous study we have shown that partial buffer saturation can be a mechanism of facilitation (Rozov et al., 2001). However, in that study facilitation was artificially induced by loading the presynaptic cell with moderate concentrations (0.02–0.7 mM) of the fast Ca^{2+} buffer BAPTA ($k_{on} = 4 \times 10^8\ M^{-1}\ s^{-1}$; Naraghi and Neher, 1997). In contrast to facilitation due to accumulation of residual Ca^{2+}, BAPTA-induced facilitation was not blocked by the slow Ca^{2+} buffer EGTA ($k_{on} = 2.5 \times 10^6\ M^{-1}\ s^{-1}$; Naraghi and Neher, 1997). However, whether partial buffer saturation is of physiological relevance remained unclear. This issue is particularly interesting, considering that a number of neuronal populations express fast endogenous Ca^{2+} buffers such as CB or calretinin. In the present study we investigated the contribution of partial buffer saturation to PPF in CB-containing terminals. First, we focused on CB expressing GABAergic interneurons, which form facilitating synapses onto layer 2/3 pyramidal cells of the mouse neocortex. We then extended the study to the strongly facilitating excitatory mossy fiber to CA3 pyramidal cell synapses (Henze et al., 2000; Salin et al., 1996; Toth et al., 2000), since CB is expressed in mossy fiber terminals at high concentration (Celio, 1990; Freund and Buzsaki, 1996). Finally, the contribution of CB in PPF at the studied synapses was confirmed in CB knockout mice (Klapstein et al., 1998).

Results

Washout of Endogenous Buffers from MB Cells Abolishes Paired Pulse Facilitation

We studied the contribution of Ca^{2+} buffer saturation to short-term synaptic plasticity in multipolar bursting (MB)

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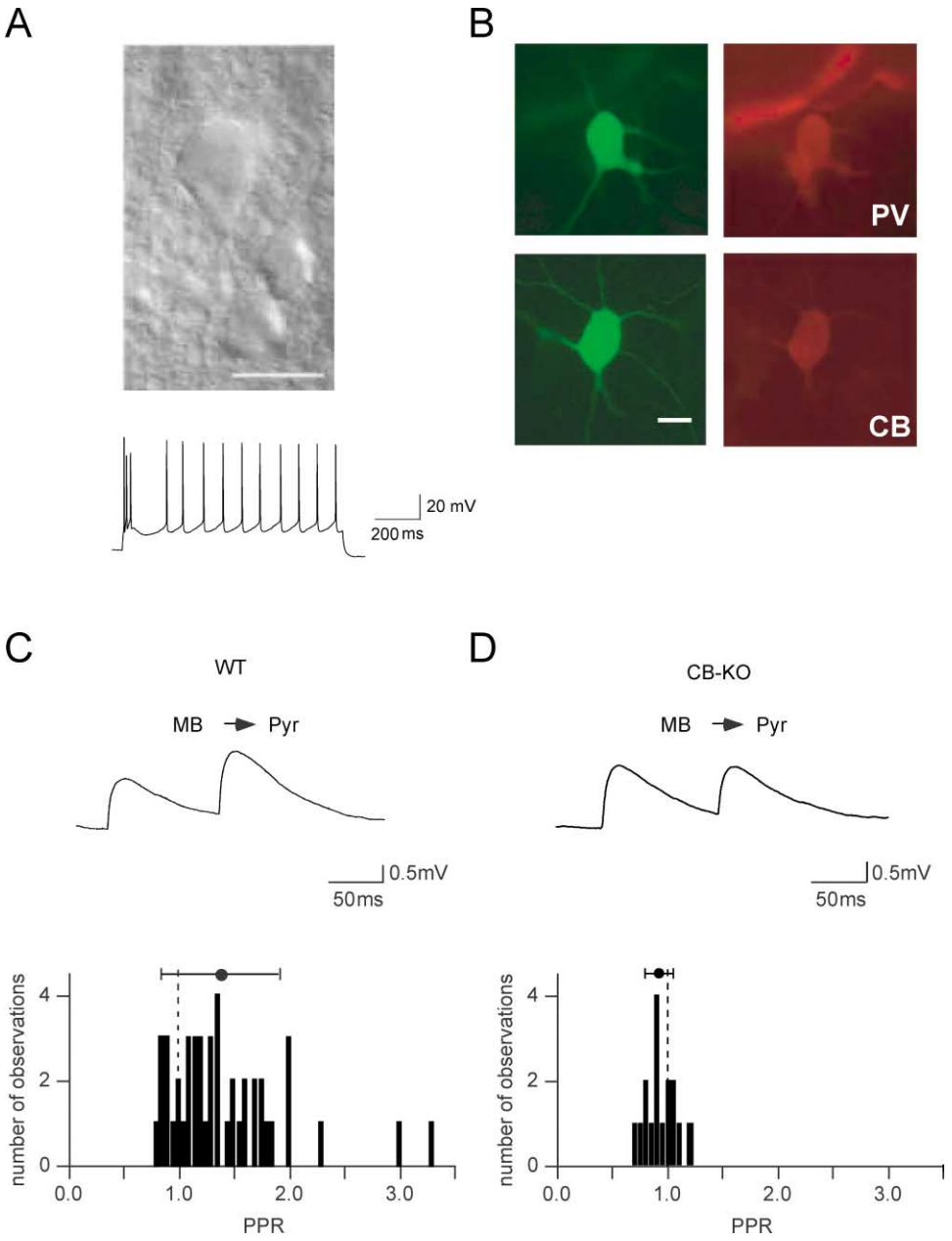

Figure 1. Morphological, Neurochemical, and Functional Signature of MB Cells

(A) Representative IR-DIC image and typical firing pattern of APs upon depolarizing current injection into a MB cell.

(B) Biocytin-filled MB cells (green, left panels) stained for the endogenous Ca^{2+} buffers PV (top right) and CB (bottom right). Scale bar in (A) and (B) equals 20 μm.

(C) Simultaneous whole-cell recordings were made from synaptically connected neurons. The upper panel shows average traces of postsynaptic responses to two APs separated by 100 ms recorded in wild-type (wt) mice. The histogram (lower panel) summarizes data from 46 experiments and shows the distribution of PPRs of IPSPs.

(D) The upper panel shows average traces of postsynaptic responses to two APs separated by 100 ms recorded in CB knockout (CB-KO) mice. The histogram (lower panel) summarizes data from 16 experiments and shows the distribution of PPRs of IPSPs. The symbols above the histograms indicate the mean ± standard deviation PPR. Only those recordings that were accomplished within 25 min after whole-cell formation of the MB cell were used for the plots.

cells, a new type of interneurons in the mouse neocortex (M. Blatow et al., submitted). MB cells can be recognized by their location close to the border between layers 1 and 2 and by their appearance in the infrared differential interference contrast (IR-DIC) video image (Figure 1A). These neurons have round or oval cell bodies, which are larger than those of layer 2/3 pyramidal cells, and have one or several visible thick dendrites. The typical resting membrane potential of MB cells is around −60 mV. Their action potential (AP) firing pattern upon prolonged depolarizing current injection is characterized by an initial burst of two or three APs followed by a

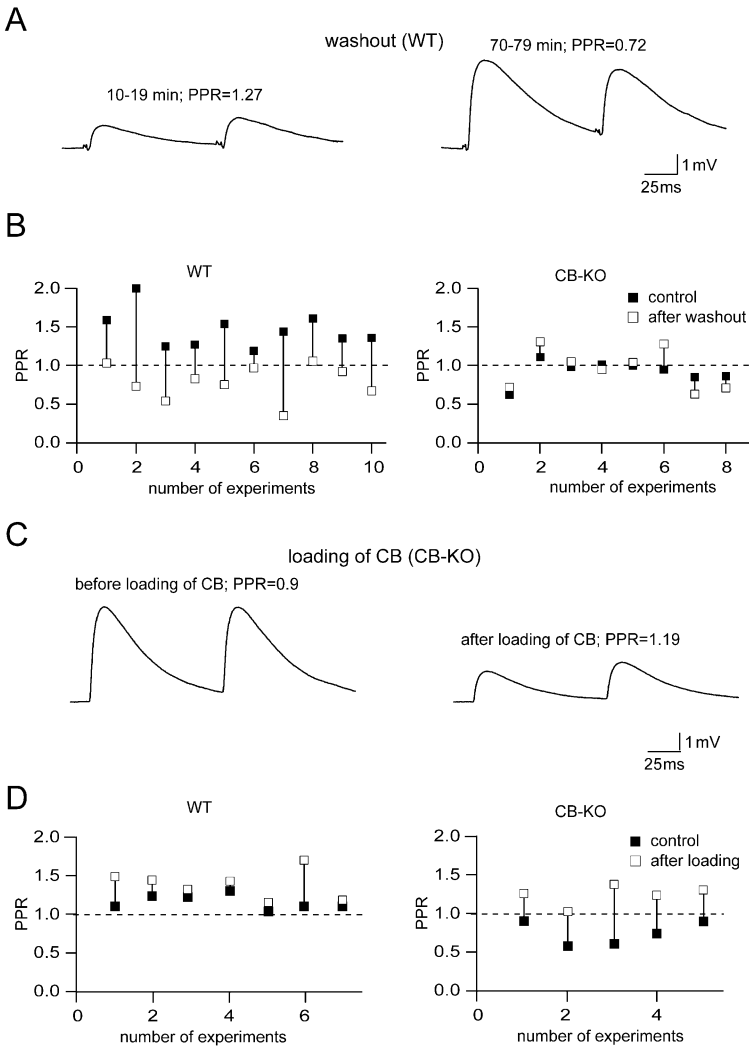

Figure 2. Short-Term Synaptic Facilitation at MB Cell to Pyramidal Cell (Pyr) Synapses

(A) Prolonged dialysis of presynaptic MB cell terminals leads to an increase of the IPSP1 amplitude and to a decrease of the PPR. Average IPSPs recorded from the same MB cell to pyramidal cell pair during the first 20 min after whole-cell formation (left trace) and 1 hr later (right trace).

(B) Washout of endogenous Ca^{2+} buffers from presynaptic MB cells significantly reduces the PPR of IPSPs in wt (left panel, n = 10) but not in CB-KO mice (right panel, n = 9). The plots show pairwise comparisons of IPSP2/IPSP1 ratios measured before (filled squares) and after (open squares) washout.

(C) Loading of CB to presynaptic MB cells in CB knockout mice reduces the amplitude of IPSP1 and restores facilitation. Average IPSPs recorded from the same MB cell to pyramidal cell pair before (left trace) and after (right trace) CB loading.

(D) Pairwise comparison of effects of CB loading to the presynaptic cell on PPR in wt (left plot) and CB-KO mice (right plot).

pronounced after hyperpolarizing gap and a subsequent train of regular spiking, low-frequency APs (Figure 1A). Immunocytochemistry revealed the coexpression of the slow Ca^{2+} buffer PV and the fast Ca^{2+} buffer CB (Figure 1B). These two endogenous mobile Ca^{2+} buffers differ substantially in their binding kinetics ($K_{on} = 4.3 \times 10^6$ M^{-1} s^{-1} for PV; $K_{on1} = 1.1 \times 10^7$ M^{-1} s^{-1} and $K_{on2} = 8.7 \times 10^7$ M^{-1} s^{-1} for CB; Lee et al., 2000; Nagerl et al., 2000). Simultaneous whole-cell recordings from pairs of connected neurons showed that inhibitory connections from MB cells to pyramidal cells in layer 2/3 of the mouse neocortex exhibited PPF of inhibitory postsynaptic potentials (IPSPs) evoked by a 10 Hz train of two APs. The average paired pulse ratio (PPR) measured as the ratio of IPSP2/IPSP1 was 1.37 ± 0.54 (n = 46; Figure 1C). Expression of either PV or CB makes accumulation of free Ca^{2+} unlikely to be the mechanism of facilitation, since either of these buffers can effectively chelate residual Ca^{2+} (Caillard et al., 2000). However, the fast Ca^{2+} buffer CB may undergo local saturation with Ca^{2+} during a train of APs and be responsible for PPF in the studied terminals. Since fast Ca^{2+} buffers determine the Ca^{2+} dynamics and release properties in the terminal, their washout should affect release probability and PPF. Indeed, in prolonged whole-cell experiments, an increase of the first response amplitude (2.57- ± 1.18-fold) was accompanied by a decrease of the PPR (from 1.46 ± 0.24 to 0.79 ± 0.23; p = 0.00004, n = 10), indicating a washout of endogenous Ca^{2+} buffers (Figures 2A and 2B, left panel). In addition, when CB (0.2 mM) was added to the pipette solution, the amplitude of the first response was decreased (0.53- ± 0.08-fold), whereas the PPR did not change significantly (from 1.19 ± 0.08 to 1.34 ± 0.14, p > 0.05, n = 7; Figure 2D, left panel). In CB knockout mice, synaptic connections between MB cells and pyramidal cells exhibited on average paired pulse depression (PPR = 0.93 ± 0.14, n = 16; Figure 1D). The PPR was significantly different from that in wild-type mice (p = 0.001). Additionally, in prolonged whole-cell experiments, no significant changes in the PPR were observed (from 0.9 ± 0.17 to 0.99 ± 0.25; n = 9, p > 0.05; Figure 2B, right panel), and the amplitude of the first response was reduced (0.72- ± 0.39-fold). Loading of CB to the presynaptic MB cell led to a reduction of the IPSP1 amplitude (0.38- ± 0.1-fold) and rescued facilitation (from 0.77 ± 0.15 to 1.24 ± 0.14; p < 0.001, n = 5; Figures 2C and 2D, right panel). In all experiments where CB was added to the intracellular

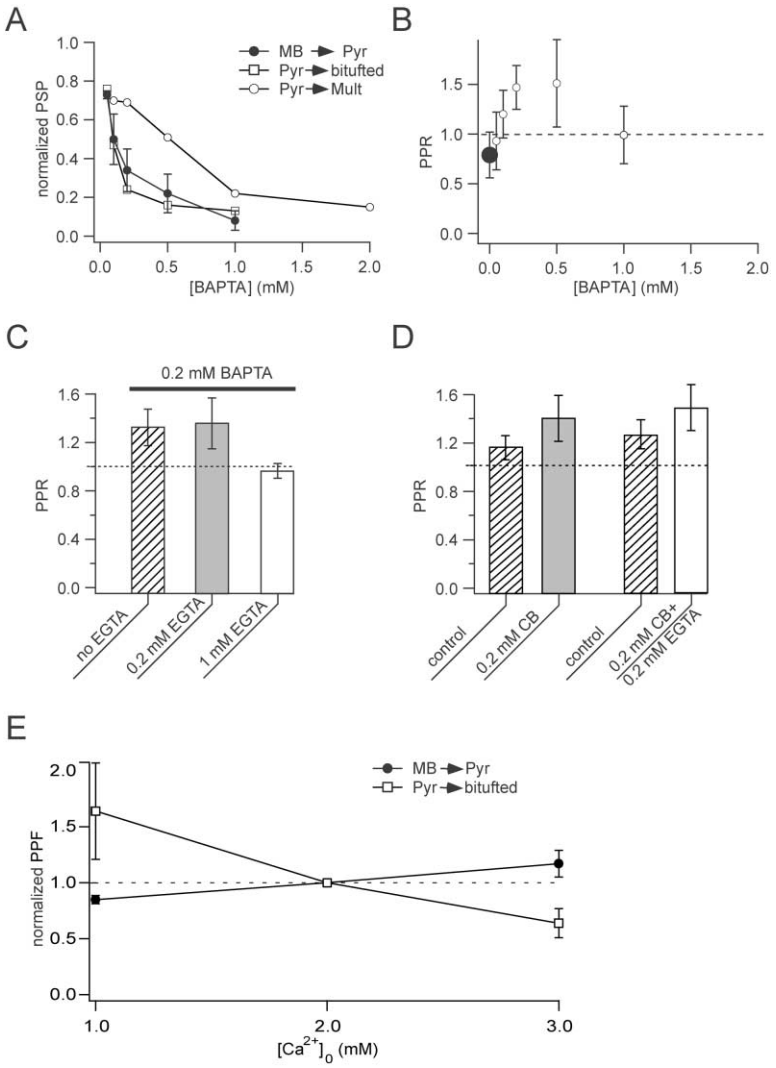

Figure 3. Effect of Exogenously Loaded Ca^{2+} Buffers on Synaptic Efficacy and PPR

(A) Concentration-dependent effect of exogenously loaded BAPTA on the mean amplitude of PSP1 in three types of synapses. The plot compares the effect of internal BAPTA on the release from predialyzed MB cell terminals on pyramidal cells (closed circles) with that previously described for a strongly facilitating (pyramidal to bitufted cells, open squares) and a strongly depressing (pyramidal to multipolar cells, open circles) type of synapse (modified from Rozov et al., 2001).

(B) Concentration-dependent effect of exogenously loaded BAPTA in predialyzed terminals on the PPR of IPSPs in MB cell to pyramidal cell connections. The PPR is plotted as a function of BAPTA concentration loaded in the presynaptic cell (open circles). The closed circle shows the average PPR after washout of endogenous Ca^{2+} buffers.

(C) EGTA at high concentration can block BAPTA-induced facilitation. PPRs of IPSPs measured in pyramidal cells after loading of presynaptic MB cells with 0.2 mM BAPTA alone and mixtures of 0.2 mM BAPTA with 0.2 mM EGTA or 1 mM EGTA, respectively.

(D) Averaged PPRs measured from MB cell to pyramidal cell pairs before (control) and after loading the MB cell with solutions containing either 0.2 mM CB or a mixture of 0.2 mM CB and 0.2 mM EGTA. Note that the presence of EGTA did not abolish facilitation.

(E) Opposite effects of extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) on PPF at two types of facilitating synapses. The graph compares the effect of different $[Ca^{2+}]_o$ on facilitation in MB cell to pyramidal cell terminals (closed circles) with that observed for the pyramidal cell to bitufted cell connection (open squares; modified from Rozov et al., 2001). In the MB to pyramidal cells connection, an increase of $[Ca^{2+}]_o$ increases facilitation, a decrease of $[Ca^{2+}]_o$ reduces facilitation. In pyramidal to bitufted cells terminals, and an increase of $[Ca^{2+}]_o$ reduces facilitation, whereas a decrease of $[Ca^{2+}]_o$ increases facilitation. In order to compare across several experiments made on different connections, PPRs were normalized to that measured in 2 mM $[Ca^{2+}]_o$.

solution, steady-state levels of IPSP amplitudes and PPRs could be reached within 40–50 min. Thus, our results suggest that CB is a causal factor for facilitation in the studied terminals.

BAPTA Rescues Paired Pulse Facilitation in Dialyzed MB Cell Terminals

It has been shown that the key element for facilitation due to buffer saturation is the competition between the Ca^{2+} buffer and the Ca^{2+} sensor for incoming Ca^{2+} during an AP (Rozov et al., 2001). Thus, the diffusional distance between Ca^{2+} channels and the Ca^{2+} sensor should be long enough so that fast Ca^{2+} buffers can effectively chelate Ca^{2+} before it reaches the release site. Testing the sensitivity of release to exogenously loaded buffers can provide valuable information about the relative distance between the Ca^{2+} sensor and Ca^{2+} channels in a given type of terminal (Neher, 1998). To probe the diffusional distance in MB cell terminals onto pyramidal cells, we tested the concentration dependence of synaptic efficacy on exogenously loaded BAPTA. In connected cell pairs, the presynaptic cell was dialyzed until the amplitude of IPSPs reached the steady-state level (30–40 min), and 100 responses were collected to establish a baseline. At this point, we considered that mobile endogenous Ca^{2+} buffers had been washed out. The same MB cell was then repatched with a pipette filled with BAPTA containing intracellular solution. After 15–20 min required for loading the buffer into the terminals, another 50–100 sweeps were collected. Figure 3A shows the BAPTA concentration-dependent reduction of the first IPSP amplitude normalized to the control IPSP amplitude (before buffer loading) in MB cell to pyramidal cell synapses ($n = 5$ for each concentration) compared to those observed in a strongly facilitating and a strongly depressing pyramidal

cell terminal (Rozov et al., 2001). The very steep concentration dependence of release on internal BAPTA in MB cell to pyramidal cell synapses was similar to that in facilitating pyramidal to bitufted cell synapses (Figure 3A) and was indicative of a long diffusional distance in both terminals. Thus, MB cell to pyramidal cell synapses have the necessary structural basis for facilitation due to Ca^{2+} buffer saturation. In addition to its strong effect on synaptic efficacy, the fast Ca^{2+} buffer BAPTA could rescue facilitation in the concentration range of 0.1–0.5 mM. At very low (0.05 mM) and very high (1 mM) buffer concentrations, the PPR was close to 1. The effects of different BAPTA concentrations on the PPR are shown in Figure 3B ($n = 5$ for each concentration). Since saturation of the fast exogenous Ca^{2+} buffer BAPTA restores facilitation in predialyzed terminals, it is likely that saturation of the fast endogenous Ca^{2+} buffer CB is responsible for facilitation under native conditions. This is supported by the reduction of the PPR in MB cell to pyramidal cell synapses in CB knockout mice (Figure 1D) and by rescue experiments with CB (Figures 2C and 2D).

It has been shown that moderate concentrations of a slow Ca^{2+} buffer can effectively prevent facilitation due to accumulation of free Ca^{2+}, but fail, however, to block facilitation due to local Ca^{2+} buffer saturation (Rozov et al., 2001). First, we tested whether this assumption is true for BAPTA-induced facilitation in MB cell to pyramidal cell terminals. Indeed, the presence of 0.2 mM EGTA in addition to 0.2 mM BAPTA in the pipette solution did not change the PPR compared to that observed with 0.2 mM BAPTA alone ($n = 3$; Figure 3C). When 1 mM EGTA was added to 0.2 mM BAPTA, facilitation was abolished ($n = 3$; Figure 3C). In these experiments, 0.2 and 1 mM EGTA were able to reduce the amplitude of the first IPSP in a concentration-dependent manner to 73% ± 20% and 43% ± 15%, respectively, despite the presence of BAPTA. This in turn implies that EGTA should have no effect on the PPR, if facilitation depends on the Ca^{2+} buffering properties of CB. When presynaptic MB cells were repatched with intracellular solutions containing either 0.2 mM CB ($n = 7$) or 0.2 mM EGTA, in addition to 0.2 mM CB ($n = 3$), amplitudes of the first IPSPs were reduced (0.53- ± 0.08-fold; 0.46- ± 0.1-fold, respectively), whereas PPRs in both cases were slightly, but not significantly, increased (from 1.26 ± 0.1 to 1.48 ± 0.19, $p > 0.05$; from 1.19 ± 0.08 to 1.34 ± 0.14, $p > 0.05$, respectively; Figure 3D).

The Ca^{2+} buffer saturation model implies that lowering extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) should reduce the saturation rate and result in reduced facilitation, whereas elevating $[Ca^{2+}]_o$ would increase the saturation rate and facilitation. Thus, PPF due to Ca^{2+} buffer saturation should be directly dependent on $[Ca^{2+}]_o$. On the other hand, the residual Ca^{2+} hypothesis predicts an inverse dependence of PPF on $[Ca^{2+}]_o$ (Katz and Miledi, 1968; Rozov et al., 2001; Zucker, 1989). To study the dependence of facilitation on $[Ca^{2+}]_o$, it was critical to prevent washout of CB from the presynaptic MB cell terminals, since it would lead to progressive changes in the IPSP1 amplitude and the PPR (see Figure 2A). Therefore, we included in these experiments 0.2 mM CB to the intracellular solution and recorded at the steady-state level. Indeed, when $[Ca^{2+}]_o$ was decreased from 2 to 1 mM, PPF at MB cell to pyramidal cell synapses was decreased. When $[Ca^{2+}]_o$ was increased from 2 to 3 mM, PPF was increased. Figure 3E compares the effect of different $[Ca^{2+}]_o$ on facilitation at MB cell to pyramidal cell synapses with that found for pyramidal to bitufted cells connections, where facilitation was shown to be due to accumulation of free Ca^{2+} (Rozov et al., 2001). Thus, taken together, our data strongly suggest that local saturation of CB during repetitive activity underlies facilitation in MB cell terminals.

Opposite Effects of Extracellular Ca^{2+} Concentration on Paired Pulse Facilitation at Two Excitatory Synapses in the Hippocampus

To substantiate our hypothesis, we extended the study to yet another strongly facilitating connection, the mossy fiber-CA3 pyramidal cell synapse, which can be reliably studied by extracellular stimulation. We chose this hippocampal synapse because it combines robust PPF (Henze et al., 2000; Salin et al., 1996; Toth et al., 2000) with a high expression of CB in dentate gyrus granule cells and mossy fiber terminals (Celio, 1990; Freund and Buzsaki, 1996). Figure 4A shows the distribution of CB immunoreactivity in the mouse hippocampus. CA3 pyramidal cells receive input not only from mossy fibers, but also from CA3 pyramidal cell (associational/commissural) collaterals. Therefore, it was important to dissect these two inputs when recording from CA3 pyramidal cells. Group II metabotropic glutamate receptors (mGluRs) are exclusively expressed on mossy fiber terminals, but not on CA3 pyramidal cell terminals (Kamiya et al., 1996; Maccaferri et al., 1998). DCG-IV, a group II mGluR agonist, substantially reduces release from mossy fiber terminals. To confirm the mossy fiber input after each experiment, 1 μM DCG-IV was added to the bath solution. We considered the connection to be mediated by mossy fibers when EPSCs were almost completely blocked by DCG-IV and by associational/commissural collaterals, when DCG-IV did not have any effect on the amplitude of EPSCs (Figure 4B).

To confirm our hypothesis that local saturation of CB underlies PPF at mossy fiber to CA3 pyramidal cell synapses, we tested the dependence of mossy fiber PPF on $[Ca^{2+}]_o$. Since mossy fiber synapses are known to undergo strong frequency facilitation (Salin et al., 1996), a stimulation rate of 0.03 Hz was chosen in these experiments to prevent progressive saturation of CB during the course of the recordings. Figure 5A (upper traces) shows that reduction of the Ca^{2+}/Mg^{2+} ratio in extracellular solution from 2 mM/2 mM to 1.5 mM/2.5 mM or 1 mM/3 mM, respectively, led to a gradual and substantial decrease of the PPR. Increase of $[Ca^{2+}]_o$ (Ca^{2+}/Mg^{2+} ratio 3 mM/1 mM), on the other hand, resulted in higher PPF at mossy fiber to CA3 pyramidal cell synapses (Figure 5B, open squares; $n = 5$). For comparison, we carried out the same experiments on Schaffer collaterals to CA1 pyramidal cell synapses, another hippocampal connection characterized by pronounced PPF. At these synapses, lowering $[Ca^{2+}]_o$ enhanced facilitation and vice versa (Figures 5A, middle traces, and 5B, filled squares; $n = 5$). In CB knockout mice, PPF at mossy fibers depended on $[Ca^{2+}]_o$ in the opposite way compared to the wild-type (Figures 5A, lower traces, and 5B, closed circles; $n = 4$). The PPR was decreased at higher and

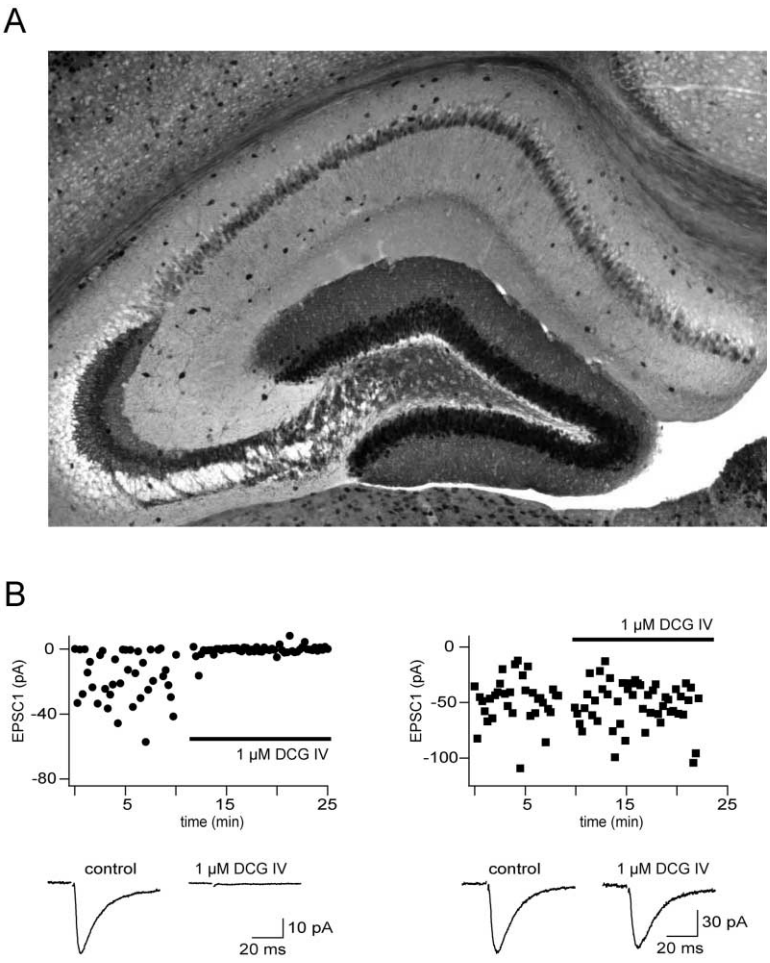

Figure 4. Immunocytochemical and Pharmacological Properties of Mossy Fiber to CA3 Pyramidal Cell Synapses

(A) Calbindin-D28k immunoreactivity in the mouse hippocampus. Note the particularly high expression in dentate gyrus granule cells and mossy fibers.

(B) The mGluR2 agonist DCG IV is used to distinguish mossy fiber (MF) from associational/commissural (AC) inputs to CA3 pyramidal cells. 1 μM DCG IV almost completely blocks release from MF terminals (left panel), but has no effect on AC synapses (right panel). The traces show average responses before and after agonist application at steady-state level.

increased at lower $[Ca^{2+}]_o$, similar to PPF at Schaffer collaterals of wild-type mice. Figure 5B (right panel) compares the relative effect of different $[Ca^{2+}]_o$ on the amplitude of the first EPSC in these three types of synapses. At all three synapses, the effect of $[Ca^{2+}]_o$ on synaptic efficacy was almost the same. These results suggest that local partial saturation of CB strongly contributes to PPF in mossy fiber terminals, whereas facilitation in Schaffer collateral terminals is most likely due to accumulation of free Ca^{2+}. A recent study (Toth et al., 2000) showed that PPF measured at mossy fibers was related to $[Ca^{2+}]_o$ in a manner consistent with accumulation of free Ca^{2+}. However, the authors measured the PPR as the ratio of the fifth and the first responses. To address the apparent discrepancy between those results and our data, we tested the effects of $[Ca^{2+}]_o$ on PPF using a five-pulse protocol. Indeed, the ratio of EPSC5/EPSC1 was decreased in 3 mM $[Ca^{2+}]_o$, as compared to 2 mM $[Ca^{2+}]_o$ (Figure 5C; n = 3). This can be explained either by a stronger contribution of vesicle depletion to the amplitude of the fifth response at higher $[Ca^{2+}]_o$ or by the oversaturation of CB during prolonged repetitive stimulation.

EGTA Blocks Paired Pulse Facilitation in Schaffer Collaterals but not in Mossy Fibers

EGTA can effectively chelate residual Ca^{2+}, preventing facilitation due to accumulation of free Ca^{2+}, but cannot determine Ca^{2+} dynamics in the presence of a faster Ca^{2+} buffer (Katz and Miledi, 1968; Rozov et al., 2001). Hence, one would expect facilitation due to buffer saturation to be insensitive to moderate EGTA concentrations, which should, however, block facilitation due to accumulation of free Ca^{2+}. Indeed, application of 100 μM EGTA-AM for 15 min (Atluri and Regehr, 1996) could completely block PPF at Schaffer collaterals (Figures 6A and 6B; n = 4). In experiments in the CA3 region, we recorded simultaneously from two adjacent pyramidal cells, assuming that the stimulation in a given place would result in responses to mossy fiber input in one of the cells, whereas the other cell most likely would receive associational/commissural input, due to the unique anatomical organization of mossy fiber axons (Acsady et al., 1998). Thus, we could compare the effect of EGTA-AM on facilitation in these two types of synapses under the same experimental conditions. After each experiment, the origin of the input was determined by application of DCGIV. In all cases, 15 min application of 100 μM EGTA-AM completely abolished facilitation in associational/commissural collaterals (n = 4), but did not have any noteworthy effect on mossy fiber PPF (Figures 6A and 6B; n = 5). However, EGTA-AM could almost completely block PPF in mossy fibers in CB knockout mice (Figures 6A and 6B; n = 3). Figure 6C summarizes the effects of EGTA-AM on the amplitude of the first EPSC in these four types of synapses.

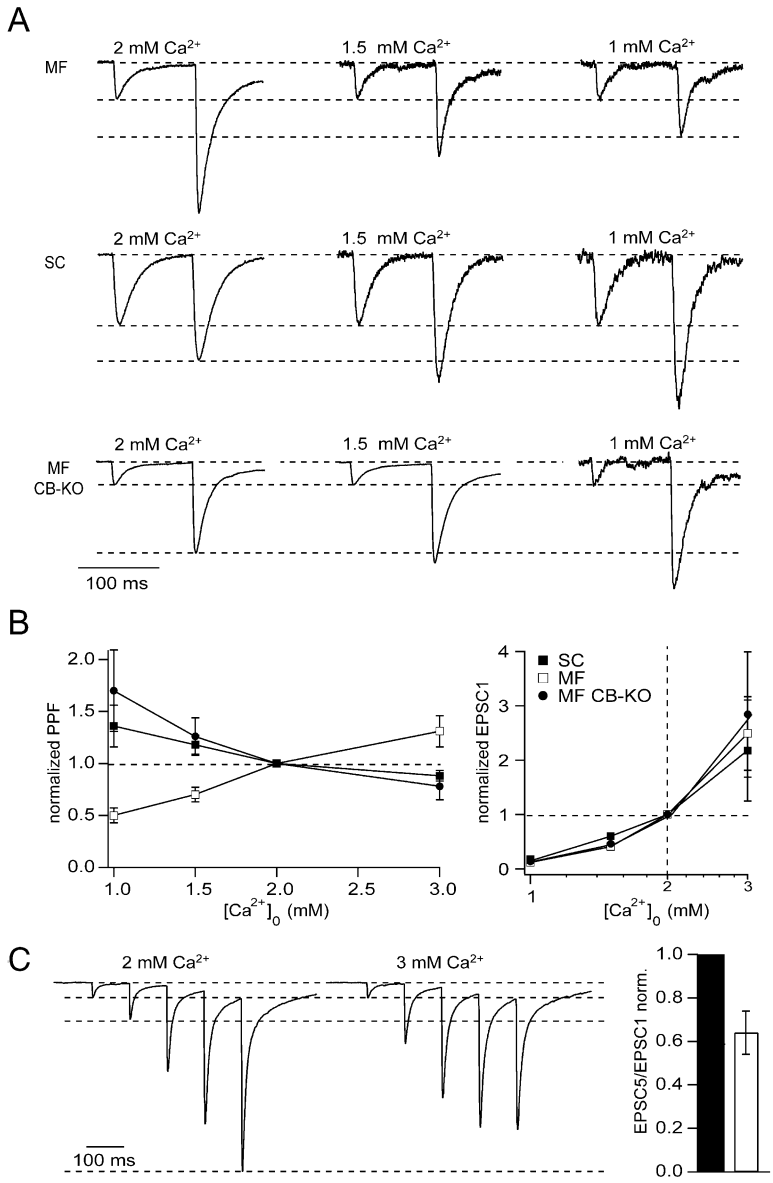

Figure 5. Opposite Effects of Extracellular Ca^{2+} Concentration ($[Ca^{2+}]_o$) on PPF at Two Excitatory Synapses in the Hippocampus

(A) EPSCs recorded from CA3 pyramidal cells during paired pulse stimulation (10 Hz) of mossy fibers in wt (MF, upper traces) and CB-KO mice (MF CB-KO, lower traces) and from CA1 pyramidal cells during paired pulse stimulation of Schaffer collaterals in wt mice (SC, middle traces) in different $[Ca^{2+}]_o$. In MF an increase of $[Ca^{2+}]_o$ increases facilitation, but a decrease of $[Ca^{2+}]_o$ reduces facilitation. In SC an increase of $[Ca^{2+}]_o$ reduces facilitation, whereas a decrease of $[Ca^{2+}]_o$ increases facilitation. In MF CB-KO, an increase of $[Ca^{2+}]_o$ reduces facilitation, and a decrease of $[Ca^{2+}]_o$ increases facilitation. The first EPSC amplitudes were normalized to allow comparison of the degree of facilitation at different $[Ca^{2+}]_o$. (B) The left graph compares the effects of changes in $[Ca^{2+}]_o$ on facilitation at MF to CA3 pyramidal cell synapses (open squares) with that at SC to CA1 pyramidal cell synapses (filled squares) and at MF to CA3 pyramidal cell synapses in CB-KO mice (closed circles). In order to compare across several experiments made on different connections, PPRs were normalized to that measured in 2 mM $[Ca^{2+}]_o$. The right graph shows the dependence of EPSC1 on $[Ca^{2+}]_o$. Note that the effects of $[Ca^{2+}]_o$ on synaptic release are comparable in MF, MF CB-KO, and SC terminals. (C) EPSCs recorded from CA3 pyramidal cells upon mossy fiber stimulation with a train of five stimuli (10 Hz) in 2 and 3 mM $[Ca^{2+}]_o$. The histogram on the right shows the reduction of the EPSC5/EPSC1 ratio when $[Ca^{2+}]_o$ was raised from 2 (black bar) to 3 (white bar) mM.

Discussion

Short-term synaptic facilitation allows a rapid increase of synaptic gain in a frequency-dependent manner. PPF has been shown to be a characteristic feature of fast synaptic transmission in a number of excitatory and inhibitory connections (Gupta et al., 2000; Reyes et al., 1998; Zucker, 1999). At many excitatory synapses, PPF is believed to be due to accumulation of free Ca^{2+}. However, in synapses with a high presynaptic Ca^{2+} buffer capacity such as those containing high levels of CB, this mechanism is unlikely to operate. More recently, local Ca^{2+} buffer saturation has been proposed as a mechanism for PPF at synapses expressing fast endogenous Ca^{2+} buffers, although there was no direct evidence for this mechanism to operate under native conditions. Here we show that local and partial Ca^{2+} buffer saturation underlies PPF in at least two types of CB-containing terminals. Other fast Ca^{2+} buffers, such as calretinin, have not been detected either in MB cells or in mossy fibers. Although we cannot ultimately exclude the presence of yet another fast Ca^{2+} buffer, we conclude that CB is responsible for facilitation in the studied terminals. However, facilitation appears to be a complex process, which can be modulated at different levels and in different ways. For instance, in neocortical excitatory synapses, presynaptic metabotropic glutamate receptors have been shown to control recovery from facilitation (Sansig et al., 2001). Repetitive activation of presynaptic kainate receptor channels expressed on mossy fibers can depolarize the terminals, which, in turn, change Ca^{2+} influx and release properties (Schmitz et al., 2001). These are just two examples to illustrate the great complexity of short-term synaptic enhancement. The main goal of this study was to find out whether fast endogenous buffers can underlie PPF. Data obtained using CB knockout mice and rescue experiments support our conclusion that partial and local saturation of

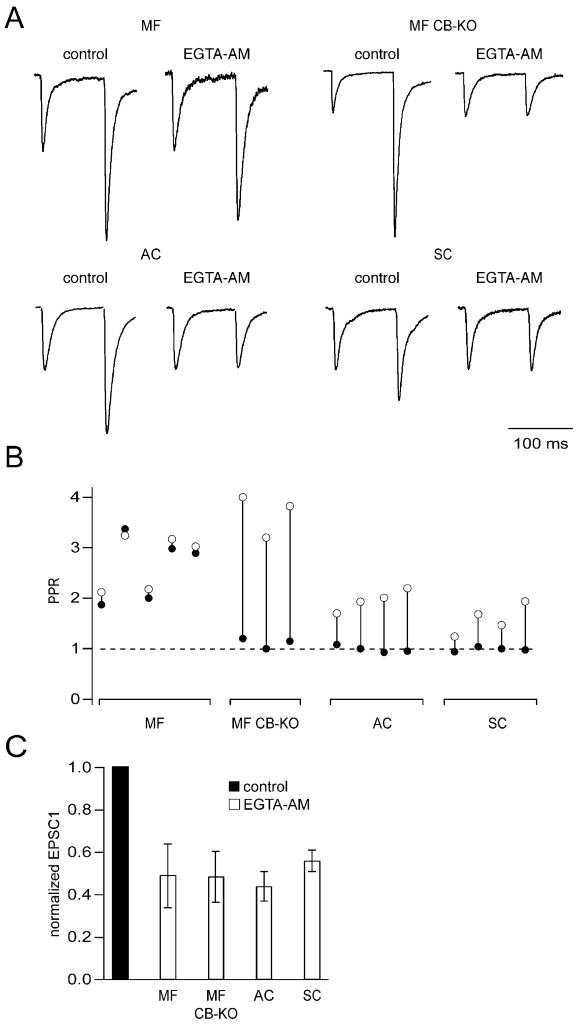

Figure 6. EGTA-AM Blocks Facilitation at Associational/Commissural (AC) and Schaffer Collateral (SC) but not at Mossy Fiber (MF) Terminals

(A) Application of 100 μM EGTA-AM for 15 min did not affect PPF at MF to CA3 pyramidal cell synapses but abolished facilitation at AC synapses and SC to CA1 pyramidal cell synapses as well as at MF to CA3 pyramidal cell synapses in CB-KO mice.

(B) Pairwise comparison of EPSC2/EPSC1 ratios in several experiments for each type of connection measured before (open circles) and after (closed circles) application of EGTA-AM.

(C) The histogram depicts the effect of EGTA-AM on the amplitude of the first response. Note that the average reduction of EPSC1 amplitude is comparable in all types of synapses.

CB strongly contributes to PPF in both MB cell and mossy fiber terminals. It is intriguing that at mossy fiber terminals, facilitation persists in CB knockout mice but has properties comparable to those predicted for facilitation due to accumulation of free Ca^{2+}. This observation is in line with the hypothesis that, independently of the mechanism of facilitation, strongly facilitating synapses should have a longer diffusional distance from Ca^{2+} channels to Ca^{2+} sensor, compared to depressing ones (Rozov et al., 2001). We also believe that this study shows an approach to distinguish between two mechanisms of facilitation.

Ca^{2+} buffer saturation-induced PPF is insensitive to moderate concentrations of EGTA, which provides a powerful tool to discriminate between two mechanisms of facilitation. However, overloading the terminal with a slow buffer can also affect the PPR (Figure 3C), most likely due to a Ca^{2+} exchange reaction between the fast and the slow buffer, when the slow buffer is present at higher concentration. This might in part explain the results reported by Salin et al. (1996), where 200 μM EGTA-AM applied for 30 min could block facilitation in mossy fibers. Under experimental conditions used here, application of 100 μM EGTA-AM for 15 min was sufficient to block facilitation in Schaffer collaterals and associated/commissural terminals but did not affect facilitation in mossy fibers. In a recent study (Kamiya et al., 2002) where a similar protocol was used, EGTA-AM also failed to block MF facilitation. The nonsignificant reduction of PPF observed by the authors can be explained by the blocking effect of EGTA-AM on AC facilitation, which contributes to the evoked responses when recording field potentials. In CB knockout mice, PPF in mossy fibers was almost completely abolished by the application of EGTA-AM. The little remaining facilitation might be explained by feedback activation of presynaptic kainate receptor channels (Schmitz et al., 2001).

MB cells express also the slow Ca^{2+} buffer PV. To address the relative contribution of PV and CB to synaptic plasticity in MB cell terminals, we "mimicked" the endogenous situation dialyzing the presynaptic MB cells with mixtures of fast (BAPTA) and slow (EGTA) Ca^{2+} buffers (Figure 3C). These results suggest that the slow Ca^{2+} buffer must be present at much higher concentration than the fast one in order to overcome facilitation due to Ca^{2+} buffer saturation, which is not the case in facilitating MB cell terminals. However, the expression of two Ca^{2+} buffers with different binding kinetics in the same presynaptic terminal might "equip" the cell with a potent mechanism for dynamic control of synaptic gain. Depending on the expression level of the slow buffer, the cell could differentially modify its type of short-term plasticity. Moreover, the expression of different Ca^{2+} buffers is not only spatially but also temporally regulated. Unlike in the adult brain where CB and calretinin are expressed in restricted neuronal populations, in the young animal their expression pattern is much broader (Alcantara et al., 1996; Jiang and Swann, 1997). This suggests that facilitation due to Ca^{2+} buffer saturation may be more frequently encountered in the developing brain. It is furthermore of note that changes in extracellular Ca^{2+} concentration have opposite effects on facilitation due to accumulation of residual Ca^{2+} and on facilitation due to Ca^{2+} buffer saturation. Differential expression of fast Ca^{2+} buffers (Alcantara et al., 1996; Jiang and Swann, 1997) taken together with the decrease of extracellular Ca^{2+} concentration during development (Jones and Keep, 1988a, 1988b) can provide a potent way for dynamic control of short-term synaptic plasticity in developing neuronal circuitries.

Experimental Procedures

Electrophysiology

Transverse neocortical and hippocampal slices of 250 μm thickness were prepared from the brains of 14-day-old C57Bl6 and CB knock-

out (Klapstein et al., 1998) mice killed by decapitation (Maccaferri and McBain, 1995; Markram et al., 1997). During recordings, slices were maintained at room temperature (22°C–24°C) in extracellular solution consisting of (in mM): 125 NaCl, 2.5 KCl, 25 glucose, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, and 1 $MgCl_2$ (pH 7.2). Cells were identified visually using infrared differential contrast video microscopy (Stuart et al., 1993). In all experiments the paired pulse protocol with an interpulse interval of 100 ms was used, unless otherwise noted. In the neocortex, whole-cell voltage recordings were performed simultaneously from pairs of synaptically connected neurons using pipettes with a resistance of 5–7 MOhm. In the hippocampus, whole-cell current recordings were made from CA1 or CA3 pyramidal cells at holding potentials of −70 mV. Synaptic responses were evoked by low-intensity stimulations (100 μs duration; 15–45 μA intensity) in the stratum lucidum of the hippocampal CA3 region or in the Schaffer collaterals region via a constant-current isolation unit (A360, World Precision Instruments, Sarasota, FL) connected to the patch pipette filled with oxygenated extracellular solution. In hippocampal experiments, 5 μM bicuculline was added to the extracellular solution to block GABAergic synaptic transmission. The stimulation rate was selected low enough to ensure recovery of the presynaptic terminals from the previous pair of stimuli (intersweep interval, 30 s). Voltage and current traces shown are averages of 50–100 sweeps. Recordings were made using an EPC-7 amplifier (HEKA Elektronik, Lambrecht, Germany). Stimulus delivery and data acquisition was performed using PULSE software (HEKA Elektronik, Lambrecht, Germany). All analysis was performed using IGOR PRO software (Wavemetrics, Lake Oswego, OR). Averaged data are given as mean ± SD.

Two intracellular solutions were used (mM): 105 K gluconate, 30 KCl, 4 Mg-ATP, 10 phosphocreatine, 0.3 GTP, and 10 HEPES (pH 7.3, KOH, 293 mOsm) for voltage recordings from both pre- and postsynaptic cells; and 120 Cs gluconate, 10 CsCl, 8 NaCl, 2 Mg-ATP, 10 phosphocreatine, 0.3 GTP, 0.2 EGTA, and 10 HEPES (pH 7.3, KOH, 293 mOsm) for current recordings. CB (Swant, Bellinzona, Switzerland) was freshly diluted in intracellular solution just before the experiment. In experiments with EGTA-AM (Molecular Probes, Eugene, OR), 10 mM EGTA was added to the intracellular solution to exclude postsynaptic effects of EGTA-AM application. EGTA-AM was freshly diluted just before the experiment. (2S,2′R,3′R)-2-(2′,3′-discarboxycyclopropyl)glycin (DCG-IV) was obtained from Tocris (Bristol, UK). Calculation of the statistical significance of differences was performed using unpaired or paired two-tailed Student's t test.

Immunocytochemistry

For neurochemical characterization of the neurons, cells were filled with biocytin (2%) dissolved in internal pipette solution. Subsequently, the slices were fixed overnight in 4% paraformaldehyde at 4°C. Fixed slices were embedded in 4% Agar and resliced at 50 μm with a vibratome. The biocytin-filled cells were visualized by FITC-conjugated avidin (3 μg/ml; Jackson Immuno Reseaerch, West Grove, PA). The procedures for the analysis using monoclonal antibodies against PV (1:1000) or CB (1:3000; Swant, Bellinzona, Switzerland) are described elsewhere (Catania et al., 1995). DAB staining was performed using the avidin-biotin-peroxydase technique with chemicals purchased from Vector Laboratories (Vectastain, ABCelite, Burlingame, CA). Control stainings for PV and CB showed no signal in pyramidal cells (n = 4) and only PV-immunoreactivity in fast-spiking cells (n = 3), excluding the possibility of filter transparence.

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References

Acsady, L., Kamondi, A., Sik, A., Freund, T., and Buzsaki, G. (1998). GABAergic cells are the major postsynaptic targets of mossy fibers in the rat hippocampus. J. Neurosci. *18*, 3386–3403.

Alcantara, S., de Lecea, L., Del Rio, J.A., Ferrer, I., and Soriano, E. (1996). Transient colocalization of parvalbumin and calbindin D28k in the postnatal cerebral cortex: evidence for a phenotypic shift in developing nonpyramidal neurons. Eur. J. Neurosci. *8*, 1329–1339.

Atluri, P.P., and Regehr, W.G. (1996). Determinants of the time course of facilitation at the granule cell to Purkinje cell synapse. J. Neurosci. *16*, 5661–5671.

Caillard, O., Moreno, H., Schwaller, B., Llano, I., Celio, M.R., and Marty, A. (2000). Role of the calcium-binding protein parvalbumin in short-term synaptic plasticity. Proc. Natl. Acad. Sci. USA *97*, 13372–13377.

Catania, M.V., Tolle, T.R., and Monyer, H. (1995). Differential expression of AMPA receptor subunits in NOS-positive neurons of cortex, striatum, and hippocampus. J. Neurosci. *15*, 7046–7061.

Celio, M.R. (1990). Calbindin D-28k and parvalbumin in the rat nervous system. Neuroscience *35*, 375–475.

Fisher, S.A., Fischer, T.M., and Carew, T.J. (1997). Multiple overlapping processes underlying short-term synaptic enhancement. Trends Neurosci. *20*, 170–177.

Freund, T.F., and Buzsaki, G. (1996). Interneurons of the hippocampus. Hippocampus *6*, 347–470.

Gupta, A., Wang, Y., and Markram, H. (2000). Organizing principles for a diversity of GABAergic interneurons and synapses in the neocortex. Science *287*, 273–278.

Henze, D.A., Urban, N.N., and Barrionuevo, G. (2000). The multifarious hippocampal mossy fiber pathway: a review. Neuroscience *98*, 407–427.

Jiang, M., and Swann, J.W. (1997). Expression of calretinin in diverse neuronal populations during development of rat hippocampus. Neuroscience *81*, 1137–1154.

Jones, H.C., and Keep, R.F. (1988a). Brain fluid calcium concentration and response to acute hypercalcaemia during development in the rat. J. Physiol. *402*, 579–593.

Jones, H.C., and Keep, R.F. (1988b). Brain interstitial fluid calcium concentration during development in the rat: control levels and changes in acute plasma hypercalcaemia. Physiol. Bohemoslov. *37*, 213–216.

Kamiya, H., Shinozaki, H., and Yamamoto, C. (1996). Activation of metabotropic glutamate receptor type 2/3 suppresses transmission at rat hippocampal mossy fibre synapses. J. Physiol. *493*, 447–455.

Kamiya, H., Ozawa, S., and Manabe, T. (2002). Kainate receptor-dependent short-term plasticity of presynaptic $Ca2+$ influx at the hippocampal mossy fiber synapses. J. Neurosci. *22*, 9237–9243.

Katz, B., and Miledi, R. (1968). The role of calcium in neuromuscular facilitation. J. Physiol. *195*, 481–492.

Klapstein, G.J., Vietla, S., Lieberman, D.N., Gray, P.A., Airaksinen, M.S., Thoenen, H., Meyer, M., and Mody, I. (1998). Calbindin-D28k fails to protect hippocampal neurons against ischemia in spite of its cytoplasmic calcium buffering properties: evidence from calbindin-D28k knockout mice. Neuroscience *85*, 361–373.

Lee, S.H., Schwaller, B., and Neher, E. (2000). Kinetics of $Ca2+$ binding to parvalbumin in bovine chromaffin cells: implications for $[Ca2+]$ transients of neuronal dendrites. J. Physiol. *525*, 419–432.

Maccaferri, G., and McBain, C.J. (1995). Passive propagation of LTD to stratum oriens-alveus inhibitory neurons modulates the temporoammonic input to the hippocampal CA1 region. Neuron *15*, 137–145.

Maccaferri, G., Toth, K., and McBain, C.J. (1998). Target-specific expression of presynaptic mossy fiber plasticity. Science *279*, 1368–1370.

Markram, H., Lubke, J., Frotscher, M., Roth, A., and Sakmann, B. (1997). Physiology and anatomy of synaptic connections between thick tufted pyramidal neurones in the developing rat neocortex. J. Physiol. *500*, 409–440.

Nagerl, U.V., Novo, D., Mody, I., and Vergara, J.L. (2000). Binding kinetics of calbindin-D(28k) determined by flash photolysis of caged Ca(2+). Biophys. J. *79*, 3009–3018.

Naraghi, M., and Neher, E. (1997). Linearized buffered Ca2+ diffusion in microdomains and its implications for calculation of [Ca2+] at the mouth of a calcium channel. J. Neurosci. *17*, 6961–6973.

Neher, E. (1998). Vesicle pools and Ca2+ microdomains: new tools for understanding their roles in neurotransmitter release. Neuron *20*, 389–399.

Rahamimoff, R. (1968). A dual effect of calcium ions on neuromuscular facilitation. J. Physiol. *195*, 471–480.

Reyes, A., Lujan, R., Rozov, A., Burnashev, N., Somogyi, P., and Sakmann, B. (1998). Target-cell-specific facilitation and depression in neocortical circuits. Nat. Neurosci. *1*, 279–285.

Rozov, A., Burnashev, N., Sakmann, B., and Neher, E. (2001). Transmitter release modulation by intracellular Ca2+ buffers in facilitating and depressing nerve terminals of pyramidal cells in layer 2/3 of the rat neocortex indicates a target cell-specific difference in presynaptic calcium dynamics. J. Physiol. *531*, 807–826.

Salin, P.A., Scanziani, M., Malenka, R.C., and Nicoll, R.A. (1996). Distinct short-term plasticity at two excitatory synapses in the hippocampus. Proc. Natl. Acad. Sci. USA *93*, 13304–13309.

Sansig, G., Bushell, T.J., Clarke, V.R., Rozov, A., Burnashev, N., Portet, C., Gasparini, F., Schmutz, M., Klebs, K., Shigemoto, R., et al. (2001). Increased seizure susceptibility in mice lacking metabotropic glutamate receptor 7. J. Neurosci. *21*, 8734–8745.

Schmitz, D., Mellor, J., Frerking, M., and Nicoll, R.A. (2001). Presynaptic kainate receptors at hippocampal mossy fiber synapses. Proc. Natl. Acad. Sci. USA *98*, 11003–11008.

Stuart, G.J., Dodt, H.U., and Sakmann, B. (1993). Patch-clamp recordings from the soma and dendrites of neurons in brain slices using infrared video microscopy. Pflugers Arch. *423*, 511–518.

Thomson, A.M. (1997). Activity-dependent properties of synaptic transmission at two classes of connections made by rat neocortical pyramidal axons in vitro. J. Physiol. *502*, 131–147.

Thomson, A.M., and Deuchars, J. (1997). Synaptic interactions in neocortical local circuits: dual intracellular recordings in vitro. Cereb. Cortex *7*, 510–522.

Thomson, A.M., Deuchars, J., and West, D.C. (1993). Single axon excitatory postsynaptic potentials in neocortical interneurons exhibit pronounced paired pulse facilitation. Neuroscience *54*, 347–360.

Toth, K., Suares, G., Lawrence, J.J., Philips-Tansey, E., and McBain, C.J. (2000). Differential mechanisms of transmission at three types of mossy fiber synapse. J. Neurosci. *20*, 8279–8289.

Winslow, J.L., Duffy, S.N., and Charlton, M.P. (1994). Homosynaptic facilitation of transmitter release in crayfish is not affected by mobile calcium chelators: implications for the residual ionized calcium hypothesis from electrophysiological and computational analyses. J. Neurophysiol. *72*, 1769–1793.

Zucker, R.S. (1989). Short-term synaptic plasticity. Annu. Rev. Neurosci. *12*, 13–31.

Zucker, R.S. (1996). Exocytosis: a molecular and physiological perspective. Neuron *17*, 1049–1055.

Zucker, R.S. (1999). Calcium- and activity-dependent synaptic plasticity. Curr. Opin. Neurobiol. *9*, 305–313.