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**Brief Communication**

# Presynaptic $Ca^{2+}$ Entry Is Unchanged during Hippocampal Mossy Fiber Long-Term Potentiation

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The hippocampal mossy fiber (MF)–CA3 synapse exhibits NMDA receptor-independent long-term potentiation (LTP), which is expressed by presynaptic mechanisms leading to persistent enhancement of transmitter release. Recent studies have identified several molecules that may play an important role in MF-LTP. These include Rab3A, RIM1$\alpha$, kainate autoreceptor, and hyperpolarization-activated cation channel ($I_h$). However, the precise cellular expression mechanism remains to be determined because some studies noticed essential roles of release machinery molecules, whereas others suggested modulation of the ionotropic processes affecting $Ca^{2+}$ entry into the presynaptic terminals. Using fluorescence recordings of presynaptic $Ca^{2+}$ in hippocampal slices, here we demonstrated that MF-LTP is not accompanied by an increase in presynaptic $Ca^{2+}$ influx during an action potential. Whole-cell recordings from CA3 neurons revealed long-lasting increases in mean frequency, but not mean amplitude, of miniature EPSCs after the high-frequency stimulation of MFs. These data indicate that the presynaptic expression mechanisms responsible for enhanced transmitter release during MF-LTP involve persistent modification of presynaptic molecular targets residing downstream of $Ca^{2+}$ entry.

*Key words: cAMP; hippocampus; long-term potentiation; mossy fiber; presynaptic $Ca^{2+}$ influx; transmitter release*

The hippocampal mossy fiber (MF) synapse provides major excitatory input onto CA3 pyramidal neurons and exhibits robust short- and long-term presynaptic plasticity (Zalutsky and Nicoll, 1990; Weisskopf and Nicoll, 1995; Kobayashi et al., 1996; Henze et al., 2000) independent of activation of NMDA receptors (Nicoll and Malenka, 1995). Because long-term potentiation (LTP) at this synapse can be induced without postsynaptic activation (Castillo et al., 1994; Mellor and Nicoll, 2001) (but see Yeckel et al., 1999) and is blocked by the inhibitors of protein kinase A (PKA), it has been proposed that a rise in cAMP concentration within the presynaptic terminals and subsequent activation of PKA are essential for induction of MF-LTP (Weisskopf and Nicoll, 1994). Recently, several studies have revealed the molecular targets of the cAMP signaling pathway. The studies of mice lacking Rab3A (Castillo et al., 1997) and RIM1$\alpha$ (Castillo et al., 2002) suggested essential roles of these two proteins in MF-LTP. Because RIM1$\alpha$ is an active zone protein that interacts with the synaptic vesicle protein Rab3A, changes in vesicular mobilization rather than modification of ion entry processes would be expected. However, this notion was challenged by the recent report by Mellor et al. (2002) showing that blockers of the hyperpolarization-activated cation channel ($I_h$), whose activity is modulated directly by cAMP, reversed already established MF-LTP. The authors proposed a hypothesis that cAMP-dependent modification of $I_h$ results in long-lasting depolarization of MF terminals. Depolarization of the terminals would enhance transmitter release by either increasing $Ca^{2+}$ entry via broadening of action potentials (Geiger and Jonas, 2000) or activating $Ca^{2+}$ channels to elevate intraterminal basal $Ca^{2+}$ levels (Turecek and Trussell, 2001). It must be noted that Chevaleyre and Castillo (2002) put through the new paper reporting strong evidence against involvement of $I_h$ in the expression of MF-LTP (see Discussion). Other lines of evidence suggesting involvement of presynaptic kainate receptors in MF-LTP (Contractor et al., 2001; Lauri et al., 2001) (but see Nicoll et al., 2000) also imply modulation of the presynaptic $Ca^{2+}$ dynamics, because kainate autoreceptors at this particular synapse was demonstrated to operate by an ionotropic mechanism (Kamiya and Ozawa, 2000; Schmitz et al., 2001).

Two possibilities suggested by these studies (i.e., modification of downstream steps involving vesicular mobilization as suggested by the studies of Rab3A and RIM1$\alpha$ knockout mice, or modulation of presynaptic $Ca^{2+}$ dynamics as suggested by involvement of $I_h$ or kainate autoreceptors) are apparently contradictory, and it has been difficult to propose the unifying model at this time. To directly determine whether $Ca^{2+}$ entry is modified, we adopted fluorescence measurement of presynaptic $Ca^{2+}$ at MF terminals (Kamiya and Ozawa, 1999) and found that action potential-driven presynaptic $Ca^{2+}$ influx is unchanged during the expres-

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sion of MF-LTP. Our results strongly support the hypothesis that the presynaptic expression mechanism responsible for MF-LTP is persistent modification of the release machinery downstream of $Ca^{2+}$ entry.

## MATERIALS AND METHODS

*Simultaneous recordings of field EPSPs and presynaptic $Ca^{2+}$.* Transverse hippocampal slices (~400 μm thick) were prepared from BALB/c mice (14–20 d of age). All experiments were performed according to the guidelines established by the Animal Care and Experimentation Committees of Gunma University and Kobe University. Slices were continuously superfused with a solution composed of the following (in mM): 127 NaCl, 1.5 KCl, 1.2 $KH_2PO_4$, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 26 $NaHCO_3$, and 10 glucose. The solution was equilibrated with 95% $O_2$ and 5% $CO_2$. Electrical stimuli (100 μsec duration, <500 μA intensity) were delivered through a tungsten concentric bipolar electrode inserted into the stratum granulosum of the dentate gyrus, and the resultant field EPSPs were recorded from the stratum lucidum in the CA3 region with glass microelectrodes of ~10 μm tip diameter filled with the standard extracellular solution (Kamiya et al., 1996). All recordings were made at room temperature (24–28°C).

Fluorescence recordings of presynaptic $Ca^{2+}$ within the mossy fiber terminals were made as described previously (Kamiya and Ozawa, 1999). Briefly, rhod-2 AM (Dojindo Laboratory, Kumamoto, Japan), a membrane-permeable $Ca^{2+}$ indicator, was loaded into the MF terminals without severing the axons. The dye was injected locally into the stratum lucidum, resulting in selective labeling of the mossy fibers. The fluorescence (excitation at 510–560 nm and monitoring above 580 nm) from the area (~100 μm diameter) containing the labeled terminals was measured with a single photodiode (S2281–01; Hamamatsu Photonics, Hamamatsu, Japan), while the field EPSPs were monitored simultaneously from the area. The $\Delta F/F$ value evoked by a single electrical stimulus was used as a measure of $[Ca^{2+}]_i$ increase during an action potential. Subtraction of the background fluorescence was not performed, because the fluorescence of unlabeled region of the slices at this wavelength was almost negligible. No attempt was made to relate the $\Delta F/F$ value to peak $[Ca^{2+}]_i$, because our methods detect the fluorescence signals from the terminals as well as the axons, and we could not estimate the relative contribution to the total signals. The output of the photodiode was *I–V* converted, amplified, and filtered at 500 Hz with an eight-pole Bessel filter (FLA-1; Cygnus Technology, Delaware Water Gap, PA). The signal was then digitized with a 12 bit analog-to-digital converter (Digidata 1200A; Axon instruments, Foster City, CA) and acquired at 10 kHz using pClamp8 software (Axon Instruments). Data in the text and figures are expressed as mean ± SEM (the number of experiments). Statistical analysis was performed using the paired *t* test unless otherwise noted, and $p < 0.05$ was accepted for statistical significance.

*Measurement of fiber volley.* The presynaptic fiber volley (FV) was recorded in the presence of 10 μM CNQX to avoid contamination of the field EPSPs. The amplitude of FV was measured as a difference between the initial positive and the following negative peaks. Field potential was filtered at 2 kHz and digitized at 20–40 kHz for FV measurement. To confirm that the responses surely reflect FV, 1 μM TTX was applied at the end of all experiments (see Figs. 1*C*, 2*C*).

*Measurement of miniature EPSCs.* Whole-cell recordings were made from CA3 pyramidal neurons, and miniature EPSCs (mEPSCs) were recorded at −70 mV in the presence of 0.5 μM tetrodotoxin and 100 μM picrotoxin (Kamiya and Ozawa, 1999). Patch pipettes were filled with an internal solution (pH 7.2) containing the following (in mM): 150 Cs gluconate, 0.2 EGTA, 8 NaCl, 10 HEPES, 2 $Mg^{2+}$ATP, and 5 QX-314 (lidocaine *N*-ethyl bromide quaternary salt). The membrane currents were filtered at 1 kHz and collected for 60 sec in each data point. The mEPSCs (6 pA amplitude threshold) was analyzed off-line using Mini Analysis Program 5.1 (Synaptosoft, Decatur, GA). No attempt was made to group the events by the rise time. The Kolmogorov–Smirnov test was used to assess the effects on amplitude and interevent interval.

## RESULTS

### Presynaptic $Ca^{2+}$ entry is unchanged by MF-LTP expression

First, we examined whether stimulus-evoked presynaptic $Ca^{2+}$ influx is modified during expression of MF-LTP. High-frequency stimulation of MF (100 Hz for 1 sec) elicited sustained potentiation of field EPSP amplitudes (195 ± 14% of control at 20 min after tetanus; $n = 8$) (Fig. 1*A*). In contrast, fluorescent signals ($F_{Ca}$) recorded simultaneously did not change significantly (94 ± 2% of control) (Fig. 1*B*). Background fluorescence ($F$), reflecting resting $Ca^{2+}$ level, was also not affected (97 ± 3% of control). The lack of effect on $F_{Ca}$ might not be attributable to saturation of indicator, because raising external $Ca^{2+}$ to 3 mM (125% of 2.4 mM standard solution) enhanced $F_{Ca}$ substantially (119 ± 2% of control; $n = 5$). In separate experiments, we monitored presynaptic FV in the presence of the AMPA receptor antagonist CNQX (10 μM), because a previous study demonstrated that tetanic stimulation produces long-lasting change in presynaptic excitability by assessing latency of FV (Mellor et al., 2002). Changes in the FV amplitude are rather small (105 ± 1% of control) at 20 min after tetanus ($n = 6$) (Fig. 1*C*). These results suggest that MF-LTP is not accompanied by a change in presynaptic $Ca^{2+}$ dynamics within each terminal but is instead attributable to enhanced efficacy of exocytotic machinery downstream from $Ca^{2+}$ influx. Then, we examined the mechanism responsible for forskolin (FSK)-induced potentiation at this synapse, which has been proposed to share common expression mechanisms with tetanus-induced LTP (Weisskopf et al., 1994). Application of FSK (50 μM for 20 min) resulted in a gradual increase in field EPSP amplitudes (480 ± 42% of the control value; $n = 6$) (Fig. 2*A*). Simultaneously recorded $F_{Ca}$ was increased by FSK (121 ±

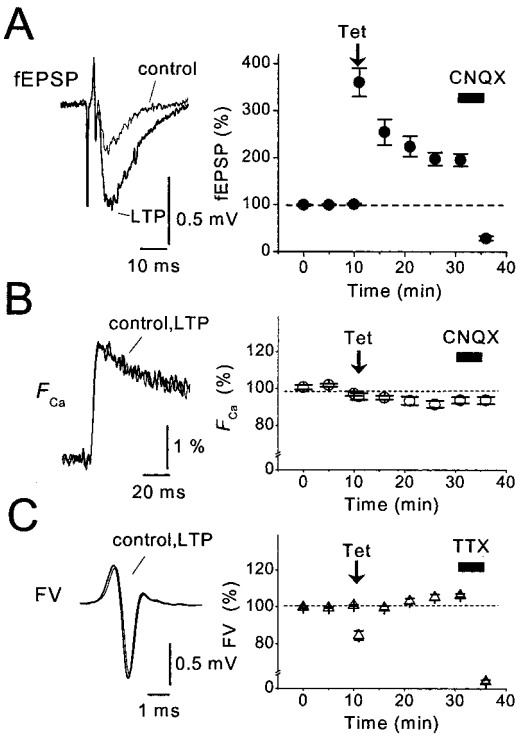


*Figure 1.* LTP at the MF–CA3 synapse is not accompanied by a change in presynaptic $Ca^{2+}$ entry. *A*, Amplitudes of field EPSP (*fEPSP*) were plotted against time. Tetanic stimulation (*Tet*; 100 Hz, 1 sec) was applied at the time shown by the *arrow*. Representative traces are those recorded before (*control*, *thin trace*) and 20 min after (*LTP*, *thick trace*) tetanic stimulation. *B*, Presynaptic $Ca^{2+}$ transients recorded simultaneously ($F_{Ca}$) were unchanged, whereas clear LTP was observed as in *A*. Application of CNQX (10 μM) did not decrease $F_{Ca}$, confirming that fluorescence signals were originated exclusively from presynaptic structures. *C*, Time course of the presynaptic FV amplitude recorded in the presence of 10 μM CNQX. The FV amplitude was decreased soon after the tetanus, although it recovered afterward.

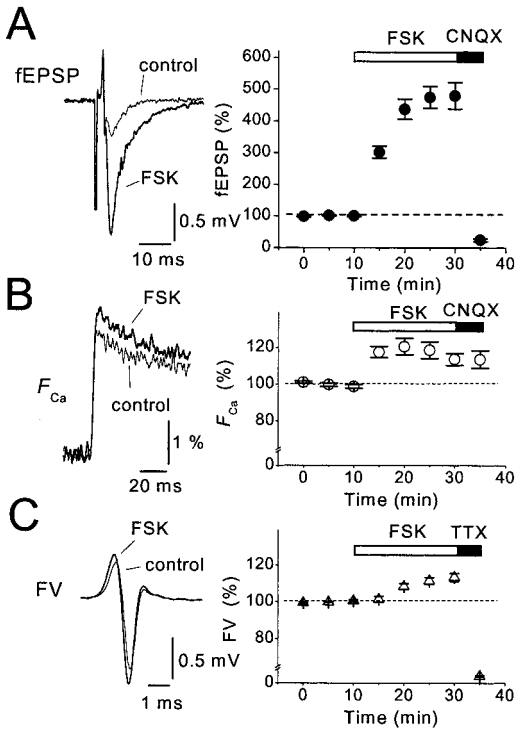


*Figure 2.* FSK-induced synaptic enhancement is independent of changes in $Ca^{2+}$ dynamics within MF terminals. *A*, Enhancement of fEPSPs during application of 50 $\mu$M FSK. *B*, Time course of $F_{Ca}$ in the same experiments as in *A*. Representative traces are those recorded before (*control*, *thin trace*) and 20 min after (*FSK*, *thick trace*) FSK application. *C*, Time course of FV amplitudes. FV was increased in size by almost the same degree as that of $F_{Ca}$, suggesting that FSK enhanced presynaptic excitability but is not accompanied by an increase in $Ca^{2+}$ influx into the individual terminals.

4% of control) (Fig. 2*B*), whereas *F* value was not affected significantly (96 ± 2% of control). FV recorded in the presence of CNQX increased in amplitude by almost the same degree as $F_{Ca}$ (115 ± 3% of control; $n = 7$; $p = 0.24$) (Fig. 2*C*). Application of 50 $\mu$M 1,9-dideoxyforskolin, an inactive analog of FSK, affected neither field EPSP amplitude (103 ± 7% of control) nor $F_{Ca}$ (94 ± 5% of control; $n = 3$). These results suggest that the FSK-induced potentiation is accompanied by the increase in the number of firing axons that explains the increase in $F_{Ca}$ during application of FSK but is unlikely attributable to an increase in action potential-driven $Ca^{2+}$ influx into the individual presynaptic terminals.

### Enhancement of frequency, but not amplitude, of miniature EPSCs during MF-LTP

To investigate changes in transmitter releasing machinery more directly, we also examined the effect of tetanic stimulation of MF on mEPSCs recorded from CA3 neurons (Jonas et al., 1993; Kamiya and Ozawa, 1999). Some events displayed relatively slow decay time course (Fig. 3$A_1$, $A_2$), possibly reflecting that kainate as well as AMPA receptors partly contribute to mEPSCs in CA3 neurons (Cossart et al., 2002). After recording control data in the presence of TTX, the TTX was removed from the perfusing solution for 10 min, and then a high-frequency stimulation (100 Hz for 1 sec) was given (Fig. 3*B*). Substantial evoked MF responses were replenished at this time (data not shown). Soon after the tetanus, TTX was added again, and 1 min records were taken every 5 min thereafter. The increase in mEPSC frequency was noted even 30 min after the tetanic stimulation (Fig. 3$A_1$) without significant changes in the distribution of the amplitudes (Fig. 3$A_3$). The cumulative amplitude histogram was not significantly affected, whereas the cumulative plot of interevent intervals showed a significant difference ($p < 0.05$; Kolmogorov–Smirnov test) (Fig. 3$A_4$). On average, mean frequency of mEPSCs increased to 212 ± 50% of the control value, whereas mean amplitude was little affected (104 ± 9% of control; $n = 14$) (Fig. 3*B*).

The frequency of mEPSCs was also increased during the application of 50 $\mu$M FSK (Fig. 3$C_1$) without significant changes in the amplitude distribution (Fig. 3$C_2$). The cumulative plot of interevent intervals, but not of amplitude, was affected by FSK ($p < 0.05$; Kolmogorov–Smirnov test) (Fig. 3$C_3$). On average, mean frequency of mEPSCs increased to 211 ± 25% of the control value, whereas mean amplitude was little affected (97 ± 5% of control; $n = 11$) (Fig. 3*D*). Application of 50 $\mu$M 1,9-dideoxyforskolin affected neither mean frequency (104 ± 9% of control) nor mean amplitude (101 ± 7% of control; $n = 6$) of mEPSCs. Because sustained elevation of basal $Ca^{2+}$ level within the MF terminals was not accompanied by LTP expression (Regehr and Tank, 1991) and we also did not observe significant change in *F* value in this study, the enhancement of frequency of mEPSCs strongly supports the notion that LTP and FSK-induced potentiation was accompanied by enhancement of the release machinery downstream from $Ca^{2+}$ entry. Taking all results together, we conclude that the expression mechanism of MF-LTP does not involve an increase in stimulation-dependent $Ca^{2+}$ influx into the terminals but is instead attributable to an increase in the efficacy of downstream exocytotic processes.

## DISCUSSION

Using fluorescence measurement of presynaptic $Ca^{2+}$ in mouse hippocampal slices, we demonstrated here that MF-LTP is not accompanied by a change in the presynaptic $Ca^{2+}$ transients, as shown for LTP at the CA1 synapses (Wu and Saggau, 1994). Instead, sustained increase in the efficacy of release machinery was suggested by the findings that mean frequency, but not mean amplitude, of mEPSCs was increased during MF-LTP.

Our results ruled out the possibility that activity-dependent broadening of the presynaptic action potential and the subsequent increase in presynaptic $Ca^{2+}$ influx (Geiger and Jonas, 2000) may underlie the expression of MF-LTP. A previous study revealed that sustained elevation of the basal $Ca^{2+}$ level within the terminals does not occur during LTP at this synapse (Regehr and Tank, 1991). Thus, MF-LTP does not involve any change in presynaptic $Ca^{2+}$ dynamics. Rather, exocytotic machinery downstream from $Ca^{2+}$ influx is selectively upregulated, as suggested by the mEPSC experiments in cultured hippocampal granule cells (Tong et al., 1996) and by the neurochemical study using MF synaptosomes (Lonart et al., 1998). $Ca^{2+}$-independent expression mechanism of MF-LTP, as demonstrated in this study, is rather unexpected in light of the recent findings showing that presynaptic $I_h$ (Mellor et al., 2002) or kainate autoreceptor (Contractor et al., 2001; Lauri et al., 2001) (but see Nicoll et al., 2000) is essential for MF-LTP, because activation of these channels would depolarize MF terminals and affect action potential-driven $Ca^{2+}$ entry processes by modulating voltage-dependent $K^+$ channels and/or $Ca^{2+}$ channels.

It should be mentioned that the very recent paper by Chevaleyre and Castillo (2002) reported strong evidence against the hypothetical roles of $I_h$ in MF-LTP. They found that organic $I_h$ blockers (ZD7288 and DK-AH269), which had been supposed to

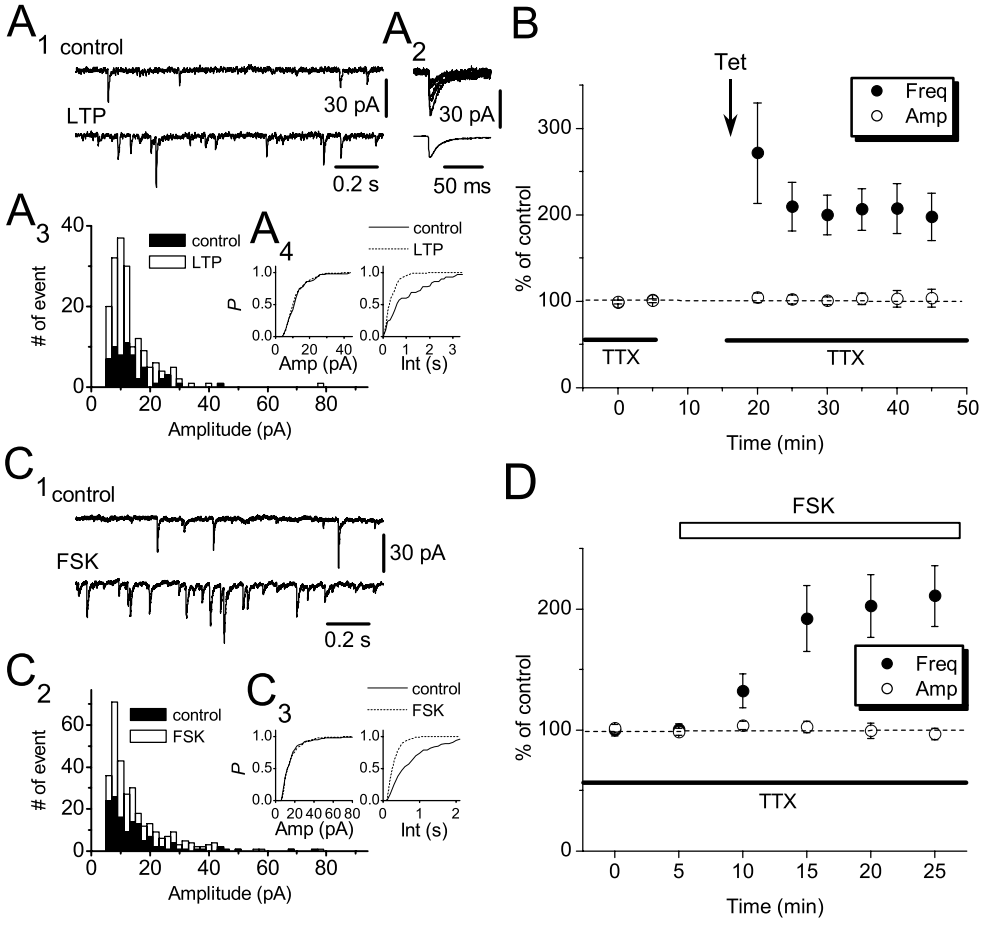


*Figure 3.* Long-lasting enhancement of frequency of mEPSCs during MF-LTP (*A*, *B*) and FSK-induced enhancement (*C*, *D*). $A_1$, Representative traces recorded before (*control*) and 30 min after tetanic stimulation (*LTP*). $A_2$, Superimposed (*top*) and averaged (*bottom*) traces of 10 consecutive mEPSCs. $A_3$, Amplitude histograms of miniature EPSCs recorded under control conditions (*filled bars*) and 30 min after tetanus (*open bars*). $A_4$, Cumulative probability plots of amplitudes (*left*) and interevent intervals (*right*) of miniature EPSCs for control (*continuous line*) and LTP (*dotted line*) data. *B*, Averaged time course of the mean frequency (*filled circles*) or amplitude (*open circles*) of mEPSCs. Tetanic stimulation (*Tet*; 100 Hz, 1 sec) was applied at the time shown by the *arrow*. TTX (0.5 $\mu$M) was perfused during the periods as indicated. $C_1$, Representative traces recorded before (*control*) and 20 min after FSK application (*FSK*). $C_2$, Amplitude histograms of miniature EPSCs in the absence (*filled bars*) and presence (*open bars*) of 50 $\mu$M FSK. $C_3$, Cumulative probability plots of amplitudes (*left*) and interevent intervals (*right*) of miniature EPSCs for control (*continuous line*) and FSK (*dotted line*) data. *D*, Time course of the FSK effect on the mean frequency (*filled circles*) or amplitude (*open circles*).

be selective for $I_h$ channels, exert a nonspecific action to suppress MF synaptic transmission. They also performed "two-pathway" experiments to get around this masking effect of the blockers and clearly demonstrated that these blockers do not affect MF-LTP. From these results, the authors also put forward their hypothesis that MF-LTP results from a direct modification of the release machinery (Castillo et al., 1997, 2002).

Because of the negative nature of the results in this study, one may argue that saturation of the $Ca^{2+}$ indicator would mask the changes in the $Ca^{2+}$ transients. However, we tried to minimize this possible artifact by using the relatively low-affinity $Ca^{2+}$ indicator rhod-2 (Minta et al., 1989) instead of the higher-affinity dye fura-2. In fact, the signals increased substantially by several conditions, e.g., application of phorbol ester (Honda et al., 2000), paired stimuli at short intervals (Kamiya et al., 2002), or elevated external $Ca^{2+}$ concentration (this study). We also paid attention to load the dye at room temperature to reduce the compartmentalization into the mitochondria. As a result, the signal displays monotonic decay after the peak (Kamiya and Ozawa, 1999; Kamiya et al., 2002), suggesting minimal contribution of the signal originated from mitochondria or other organelles.

To examine the changes in the efficacy of release machinery, we examined the mEPSCs recorded from CA3 neurons. Because we cannot distinguish the origin of the observed minis (MF terminals or the other presynaptic terminals making contact on CA3 neurons), contamination of those originated from non-MF inputs might distort the present results in an unevaluated way. Therefore, it must be emphasized that the effects of tetanic stimulation or FSK might be somewhat underestimated, because these manipulations are expected to selectively affect minis originated from MF terminals (Weisskopf et al., 1994). More importantly, however, it certainly supports the notion that MF-LTP and FSK potentiation are accompanied by enhanced efficacy of release machinery at the MF terminals.

FV amplitude was increased by application of FSK but not by LTP-inducing tetanic stimulation. These findings might be interpreted as follows. The excitability of MF axons is enhanced by both manipulations via cAMP elevation in the MF terminals (Mellor et al., 2002). Bath application of FSK would raise cAMP levels in the whole population of MF terminals and thus increased fiber volley amplitude by recruitment of surrounding subthreshold fibers. In contrast, tetanic stimulation might elevate cAMP concentration only within the stimulated MF terminals and therefore does not lead to increase in the number of stimulated axons. Differential effects of tetanic stimulation and FSK on FV amplitude would be important for answering the question of why FSK caused robust enhancement of MF responses in knock-out mice of Rab3A (Castillo et al., 1997), RIM1$\alpha$ (Castillo et al., 2002), RI$\beta$ and C$\beta_1$ isoforms of protein kinase A (Huang et al., 1995), and type 1 adenylyl cyclase (Villacres et al., 1998) in which tetanus-induced MF-LTP is absent. Our results highlight differential mechanisms responsible for FSK-induced enhancement and tetanus LTP, and this difference may explain, at least in part, why FSK potentiated MF synaptic transmission in those mutant mice. It should be noted that FSK enhanced both FV and presynaptic $Ca^{2+}$ transient to the same degree in the similar multifiber recordings at the parallel fiber synapses in the cerebellum

(Chen and Regehr, 1997), which also display cAMP-dependent presynaptic LTP (Salin et al., 1996).

In summary, our data clearly demonstrate $Ca^{2+}$-independent expression mechanisms for MF-LTP. Our results, together with the recent evidence reported by Chevaleyre and Castillo (2002), strongly support the hypothesis that the presynaptic expression mechanism responsible for MF-LTP is persistent modification of cellular steps involving the release machinery of synaptic vesicles downstream from $Ca^{2+}$ influx.

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