

# Ca<sup>2+</sup> influx-independent synaptic potentiation mediated by mitochondrial Na<sup>+</sup>-Ca<sup>2+</sup> exchanger and protein kinase C

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Activity-dependent modulation of synaptic transmission is an essential mechanism underlying many brain functions. Here we report an unusual form of synaptic modulation that depends on Na<sup>+</sup> influx and mitochondrial Na<sup>+</sup>-Ca<sup>2+</sup> exchanger, but not on Ca<sup>2+</sup> influx. In Ca<sup>2+</sup>-free medium, tetanic stimulation of *Xenopus* motoneurons induced a striking potentiation of transmitter release at neuromuscular synapses. Inhibition of either Na<sup>+</sup> influx or the rise of Ca<sup>2+</sup> concentrations ([Ca<sup>2+</sup>]<sub>i</sub>) at nerve terminals prevented the tetanus-induced synaptic potentiation (TISP).

Blockade of Ca<sup>2+</sup> release from mitochondrial Na<sup>+</sup>-Ca<sup>2+</sup> exchanger, but not from ER Ca<sup>2+</sup> stores, also inhibited TISP. Tetanic stimulation in Ca<sup>2+</sup>-free medium elicited an increase in [Ca<sup>2+</sup>]<sub>i</sub>, which was prevented by inhibition of Na<sup>+</sup> influx or mitochondrial Ca<sup>2+</sup> release. Inhibition of PKC blocked the TISP as well as mitochondrial Ca<sup>2+</sup> release. These results reveal a novel form of synaptic plasticity and suggest a role of PKC in mitochondrial Ca<sup>2+</sup> release during synaptic transmission.

## Introduction

Activity-dependent modulation of synaptic transmission is a fundamental mechanism for the development and functions of the brain. Information processing, which is coupled tightly with firing of neuronal action potentials at different frequencies, often leads to changes in the efficacy of transmission lasting for a short or long period of time, a general phenomenon known as synaptic plasticity. Experimentally, this could be mimicked by repetitive stimulation of presynaptic neurons. Long-term forms of synaptic plasticity that last for at least 1 h, such as hippocampal long-term potentiation (LTP), are mediated mostly by postsynaptic mechanisms (Luscher et al., 2000). Short-term forms of synaptic plasticity lasting from seconds to minutes are often due to changes in presynaptic transmitter secretion (Zucker and Regehr, 2002). At the neuromuscular junction (NMJ), a brief, high-frequency stimulation results in an enhancement of transmitter release that lasts many minutes, called post-tetanic potentiation (PTP). Recently, tetanic stimulation has also been shown to

induce a long-lasting enhancement of transmission similar to LTP at the neuromuscular synapses in *Xenopus* nerve-muscle cultures (Wan and Poo, 1999).

Although it is generally believed that presynaptic forms of plasticity are due to a prolonged elevation of intracellular concentrations of free Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) after the termination of tetanus, the precise molecular mechanisms for the enhancement of transmitter secretion remain unclear. At the resting nerve terminals, [Ca<sup>2+</sup>]<sub>i</sub> is generally <100 nM. This is accomplished by the Ca<sup>2+</sup> ATPase, which effectively pumps Ca<sup>2+</sup> out of the terminals, and by the plasmalemmal Na<sup>+</sup>-Ca<sup>2+</sup> exchanger, which allows entry of three Na<sup>+</sup> in exchange for the efflux of one Ca<sup>2+</sup> (Blaustein and Lederer, 1999; Garcia and Strehler, 1999). The key step in triggering transmitter secretion is an elevation of terminal [Ca<sup>2+</sup>]<sub>i</sub>. This could be achieved by a number of mechanisms. First, a well-established mechanism is the action potential-driven

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Abbreviations used in this paper: CaMKII, Ca<sup>2+</sup>/calmodulin-dependent kinase II; CGP, CGP37157; CheT, chelerythrine; DRG, dorsal root ganglion; ESC, evoked synaptic current; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenyl hydrazone; GF, GF 1092303X; IP3, inositol 1,4,5-trisphosphate; LTP, long-term potentiation; NMDG, *N*-methyl-D-glucamine; NMJ, neuromuscular junction; PTP, post-tetanic potentiation; SSC, spontaneous synaptic current; TISP, tetanus-induced synaptic potentiation; TTX, tetrodotoxin; XeC, Xestospongion C.

membrane depolarization, leading to  $\text{Ca}^{2+}$  influx through voltage-gated  $\text{Ca}^{2+}$  channels. The second and more complex mechanism is the release of  $\text{Ca}^{2+}$  from intracellular organelles. One class of such organelles is the ER. Two types of ligand-gated  $\text{Ca}^{2+}$  channels are involved in  $\text{Ca}^{2+}$  release from the ER: the IP3 receptor, operated by inositol 1,4,5-trisphosphate (IP3), and the ryanodine receptor, gated by  $\text{Ca}^{2+}$  as well as cyclic ADP ribose (Berridge, 1998). Although still a fairly new concept, transmitter secretion triggered or modulated by  $\text{Ca}^{2+}$  release from the ER has been shown in a number of synapses (Smith and Cunnane, 1996; Cochilla and Alford, 1998; Mothet et al., 1998; Yang et al., 2001). The other class of organelles is mitochondria, which represents a transient storage mechanism for  $\text{Ca}^{2+}$ . An accumulation of  $\text{Ca}^{2+}$  in the mitochondria induced by certain stimuli is released when the stimulus is terminated (Tang and Zucker, 1997; Melamed-Book and Rahamimoff, 1998). Under physiological conditions, mitochondrial  $\text{Ca}^{2+}$  release is achieved primarily by the  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger on the mitochondrial membranes. It has been recently shown that the massive secretion of transmitters at the NMJ induced by  $\alpha$ -latrotoxin is mediated by the mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger (Tsang et al., 2000). Finally, when cells are overloaded with  $\text{Na}^+$  and extracellular  $\text{Ca}^{2+}$  is high, the plasmalemmal  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger may operate in a "reverse mode" to allow  $\text{Ca}^{2+}$  entry into the cells (Zhong et al., 2001).

Many forms of activity-dependent synaptic plasticity require  $\text{Ca}^{2+}$  influx. Using a cultured neuromuscular synapse preparation in which  $\text{Ca}^{2+}$  influx has been completely prohibited, we report here a novel form of synaptic plasticity that would be difficult to reveal in normal circumstances. A train of tetanic stimulation induces a robust potentiation of neurotransmitter release, as well as an increase in  $[\text{Ca}^{2+}]_i$ , at the developing NMJ in the absence of extracellular  $\text{Ca}^{2+}$ . Detailed analyses using both pharmacological and molecular approaches indicate that this synaptic potentiation is independent of  $\text{Ca}^{2+}$  release from ER ryanodine or IP3 receptors, but requires  $\text{Na}^+$  influx. The increase in  $\text{Na}^+$  concentration in the nerve terminals triggers  $\text{Ca}^{2+}$  efflux through the mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger, leading to the tetanus-induced synaptic potentiation (TISP). In addition, inhibition of PKC dramatically attenuated TISP as well as mitochondrial  $\text{Ca}^{2+}$  release. We also show that blockade of the mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger inhibits the synaptic potentiation and  $[\text{Ca}^{2+}]_i$  increase in normal extracellular  $\text{Ca}^{2+}$ . Thus, this form of synaptic plasticity may occur during the bursting activity at the NMJ in vivo. Our studies may also help understand the contribution of mitochondria and PKC in transmitter release and provide a useful model to investigate molecular mechanisms for transmitter release without the interference of  $\text{Ca}^{2+}$  influx.

## Results

### TISP independent of $\text{Ca}^{2+}$ influx

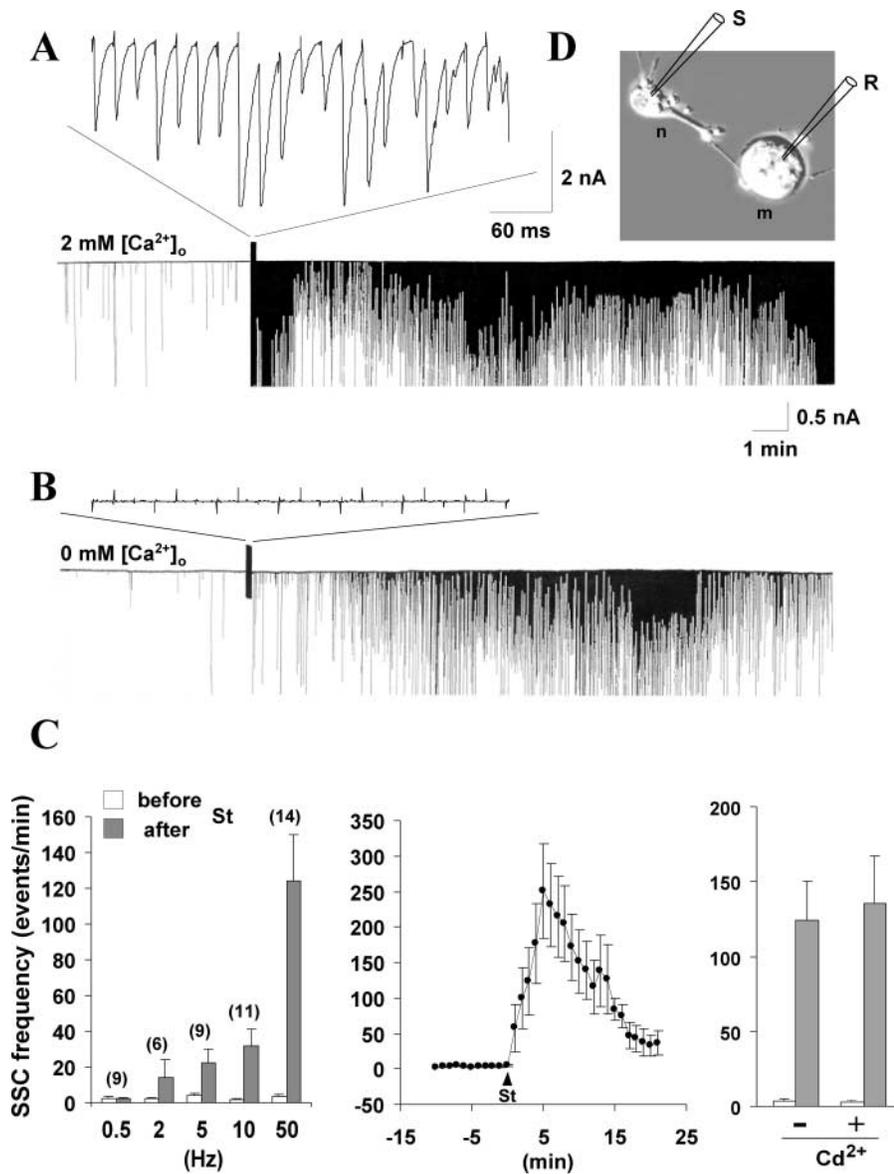
Spontaneous synaptic currents (SSCs) were recorded from innervated myocytes in 1-d-old *Xenopus* nerve-muscle cocultures (e.g., Fig. 1 D) under whole-cell, voltage-clamp conditions. Stimulation of the presynaptic motoneurons with a train of repetitive, high-frequency stimuli (or tetanus, 50

Hz, 10 s) elicited a striking potentiation of synaptic transmission. The frequency of SSCs increased more than 100 times immediately after the tetanus (Fig. 1 A). The average amplitudes of SSCs before and after the tetanus were not significantly different, suggesting that this form of synaptic plasticity is due primarily to an enhancement of presynaptic transmitter release (unpublished data). Surprisingly, the same tetanus induced a very similar synaptic potentiation in the complete absence of  $\text{Ca}^{2+}$  influx.  $\text{Ca}^{2+}$ -free conditions were achieved by using medium containing 0 mM extracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_o$ ) plus 3 mM EGTA. Under these conditions, tetanus still elicited a marked enhancement of synaptic transmission (Fig. 1 B). Similar results were obtained in zero  $[\text{Ca}^{2+}]_o$  plus 0.4 mM  $\text{Cd}^{2+}$  to block all voltage-gated  $\text{Ca}^{2+}$  channels (Fig. 1 C, right). The average frequency of SSCs increased by more than 60-fold. The onset of the potentiation was slightly slower (Fig. 1 B). It is important to note that whereas the tetanic stimulation induced evoked synaptic currents (ESCs) of various amplitudes in normal medium (Fig. 1 A, inset), the same tetanus elicited absolutely no ESCs in  $\text{Ca}^{2+}$ -free medium (Fig. 1 B, inset).

Detailed analysis indicated that with the fixed stimulation duration (10 s), the magnitude of synaptic potentiation depended on the frequency of the stimulation. No potentiation was observed at 0.5 Hz, and an  $\sim 10$ -fold increase in SSC frequency could be induced by stimulation at 2–10 Hz (Fig. 1 C, left). At 50 Hz, the synaptic potentiation reached a new level, and stimulation frequency  $> 50$  Hz did not induce further potentiation (unpublished data). In medium containing normal  $[\text{Ca}^{2+}]_o$ , synaptic efficacy was increased almost immediately after the application of the tetanus (Fig. 1 A). In  $\text{Ca}^{2+}$ -free medium, the SSC frequency began to rise  $\sim 1$ –2 min after the tetanus, and the maximal enhancement was usually observed  $\sim 5$ –10 min later (Fig. 1 C, middle). After the peak, the SSC frequency gradually decreased, but it never returned to baseline for as long as the recording can be held (up to 60 min).

### Dependence on $\text{Na}^+$ influx and the rise of intracellular $\text{Ca}^{2+}$

Two effects are elicited by tetanic stimulation of presynaptic neurons in normal conditions: repetitive firing of high frequency action potentials and large influx of  $\text{Ca}^{2+}$ . As the TISP was completely independent of  $\text{Ca}^{2+}$  influx, we tested the role of action potentials in this unusual form of plasticity. Firing of action potentials results from a rapid and large  $\text{Na}^+$  influx, followed by a delayed efflux of  $\text{K}^+$  ions. To determine whether the TIPS is mediated by  $\text{Na}^+$  influx, we reduced  $\text{Na}^+$  concentration in the extracellular medium ( $[\text{Na}^+]_o$ ) by half (from 115 mM to 57.5 mM), by replacing  $\text{Na}^+$  with *N*-methyl-D-glucamine (NMDG) (Simasko, 1994). Patch recordings were made on the nerve terminals distal to the synapses made between motor axons and muscle cells (Fig. 2 A). At this low  $[\text{Na}^+]_o$ , electric stimulation could still reliably induce action potentials, with lower amplitudes (Fig. 2 C). Moreover, action potentials could be recorded at these terminals during the entire course of the tetanic stimulation, suggesting that action potentials fully invaded the presynaptic terminals (Fig. 2 B). Under the low  $[\text{Na}^+]_o$  conditions, however, the effect of tetanus was dra-



**Figure 1. TISP in the absence of Ca<sup>2+</sup> influx.** SSCs were recorded under whole-cell, voltage-clamp conditions ( $V_h = -70$  mV) from neuromuscular synapses in *Xenopus* nerve-muscle cultures. A train of tetanic stimulation was applied to the presynaptic motoneurons to induce synaptic potentiation. (A) A typical recording showing that a 10-s, 50-Hz stimulation results in an immediate and massive increase in SSC frequency in normal medium. Inset shows ESCs induced by tetanic stimulation at a much higher time resolution. (B) An example showing that the same stimuli induced a slightly delayed increase in SSC frequency in Ca<sup>2+</sup>-free medium. The inset shows a complete lack of ESCs in the absence of extracellular Ca<sup>2+</sup>. (C, left) Frequency dependence of TISP. The presynaptic neurons were stimulated for 10 s at different frequencies as indicated. Note that a 50-Hz stimulation gives rise to the maximal synaptic potentiation. Unless indicated otherwise, this and all other figures show experiments done in Ca<sup>2+</sup>-free medium. SSC frequency was calculated by averaging from a 10-min recording immediately before the tetanus and at the peak. The numbers of experiments are indicated above the columns. (C, middle) Time course of TISP.  $n = 14$ . "St" in this and all other figures means application of tetanus (10 s, 50 Hz). (C, right) Quantification of TISP in the presence and absence of Cd<sup>2+</sup> (0.4 mM). SSC frequencies were measured before and 8 min after tetanus. (D) A Hoffman microscope image showing a motoneuron (n) innervating a myocyte (m) in the nerve-muscle coculture. S, stimulating electrode; R, recording electrode.

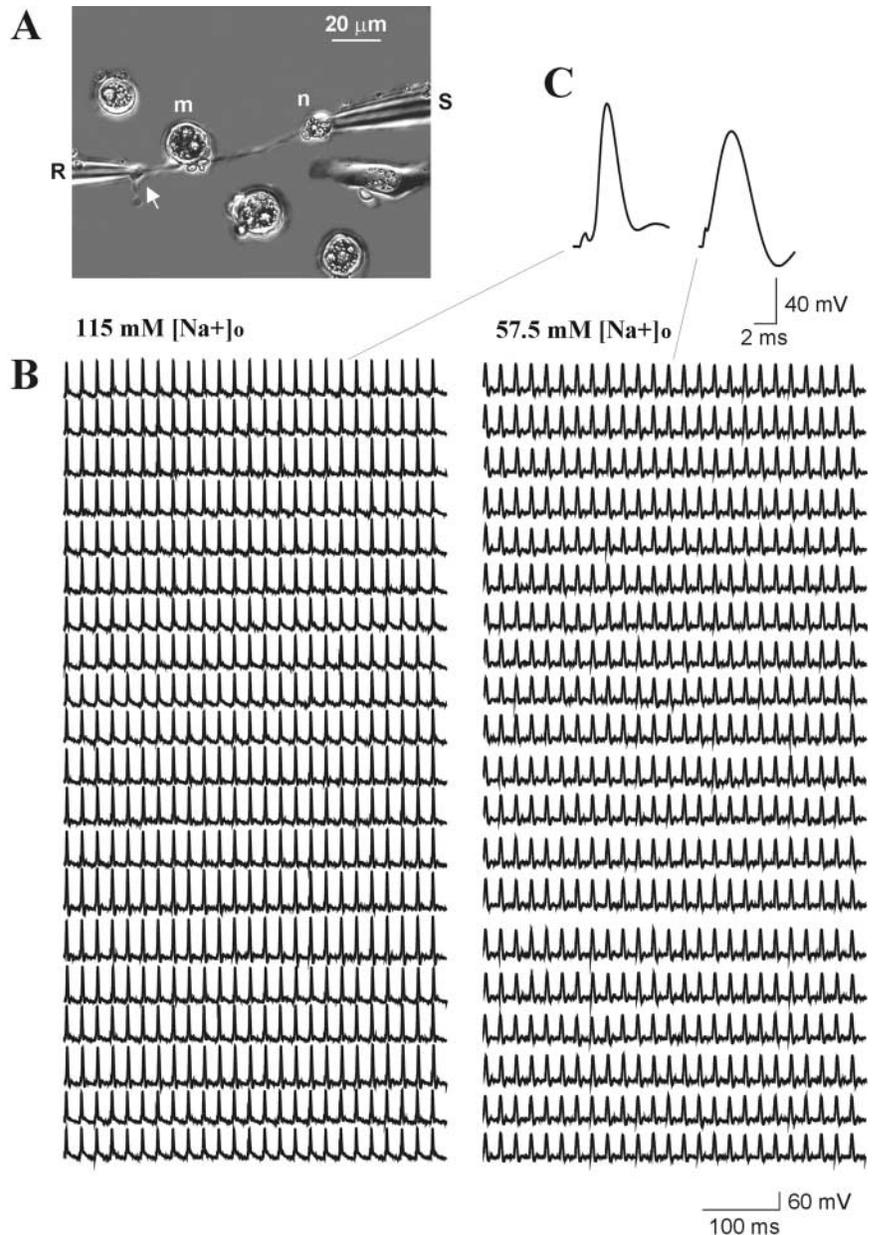
matically reduced (Fig. 3 A). SSC frequency increased by 68-fold after tetanic stimulation in normal Na<sup>+</sup> medium, but showed only fivefold increase when [Na<sup>+</sup>]<sub>o</sub> was reduced to 57.5 mM (Fig. 3 B). To test whether Na<sup>+</sup> influx into the nerve terminals is required for TISP, we inhibited Na<sup>+</sup> channels at the terminals by rapid perfusion of tetrodotoxin (TTX, 0.5 μM) to a very restricted area around the nerve terminals. TTX at this concentration completely blocked Na<sup>+</sup> channels, and therefore action potentials, in the spinal neurons (unpublished data). TTX was applied through a fine glass pipette positioned near the synapse under recording by gravity. A suction pipette was placed in the opposite side of the perfusion pipette to remove excess TTX. This method has been shown to restrict drug exposure to a very small area at terminals/axons (Stoop and Poo, 1995). As shown in Fig. 3 C, application of tetanus during TTX perfusion elicited virtually no potentiation, whereas subsequent application of the same tetanus induced a robust potentiation in the same neuron during perfusion of Ringer solution. Quantitative analysis indicated that local TTX perfusion vir-

tually prevented the tetanus-induced potentiation (Fig. 3 D). A similar brief perfusion of Ringer solution to naïve synapses had no effect on TISP (Fig. 3 D). These results strongly suggest that Na<sup>+</sup> entry into the nerve terminals is important for TISP.

In addition to Na<sup>+</sup> influx, propagation of action potential to the nerve terminals elicits K<sup>+</sup> efflux, leading to extracellular K<sup>+</sup> accumulation. Two experiments were performed to address this possibility. (1) To prevent extracellular K<sup>+</sup> accumulation, we perfused Ca<sup>2+</sup>-free Ringer solution to the neuromuscular synapses. Tetanic stimulation induced the same magnitude of synaptic potentiation while the synapses were continuously perfused by Ca<sup>2+</sup>-free Ringer solution (e.g., Fig. 3 C). (2) To mimic the extracellular K<sup>+</sup> accumulation, we perfused high K<sup>+</sup> solution locally at the synapses. This manipulation did not cause an increase in synaptic efficacy in Ca<sup>2+</sup>-free medium (Fig. 4 B). Thus, extracellular K<sup>+</sup> accumulation does not appear to be involved in TISP.

Direct membrane depolarization has recently been shown to induce transmitter release in the cell bodies of dorsal root

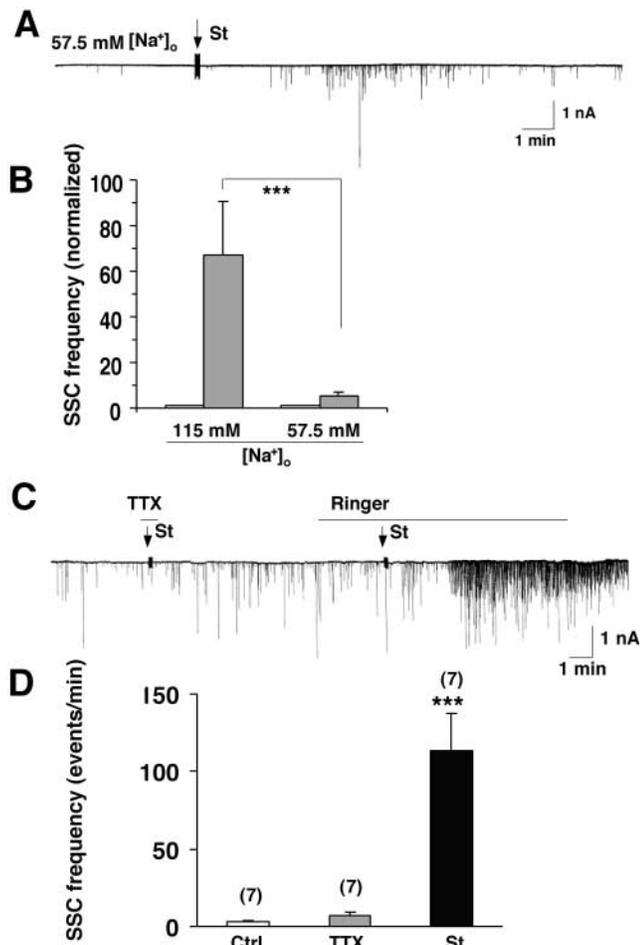
**Figure 2. Propagation of action potentials to the nerve terminals.** (A) Light microscopic image showing the cultured cells and electrodes. A stimulation electrode (S) was attached to the neuronal soma (n). Patch recording (R) was made under current clamp conditions on the nerve terminal (arrow) distal to the synapse made between a motor axon and a myocyte (m). (B) An entire train of tetanus-induced action potentials recorded from a nerve terminal in  $\text{Ca}^{2+}$ -free medium in regular  $[\text{Na}^+]_o$  (115 mM, left) and low  $[\text{Na}^+]_o$  (57.5 mM, right). (C) Enlarged traces of action potentials in regular and low  $[\text{Na}^+]_o$  conditions.



ganglion (DRG) neurons (Zhang and Zhou, 2002). To test whether TISP is due to direct depolarization of motoneuron nerve terminals, we perfused high  $\text{K}^+$  solution locally to the terminals. After a period of control recording, a stream of high  $\text{K}^+$  solution was rapidly perfused to the synapse, leading to a local membrane depolarization of both presynaptic terminal and postsynaptic myocyte. In medium with regular extracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_o = 2 \text{ mM}$ ), depolarization of the nerve terminal triggered robust vesicle fusion at the presynaptic terminals, as shown by a cluster of synaptic currents (Fig. 4 A, inset). It also elicited a steady inward current in the voltage-clamped myocyte and, consequently, a sustained downward shift of baseline (Fig. 4 A), which returned to control levels after the removal of the high  $\text{K}^+$  pipette. In marked contrast, perfusion of the nerve terminal with high  $\text{K}^+$  solution in  $\text{Ca}^{2+}$ -free medium did not induce any transmitter release during depolarization (Fig. 4 B, inset). Under such sustained depolarization, rapid inactivation of voltage-

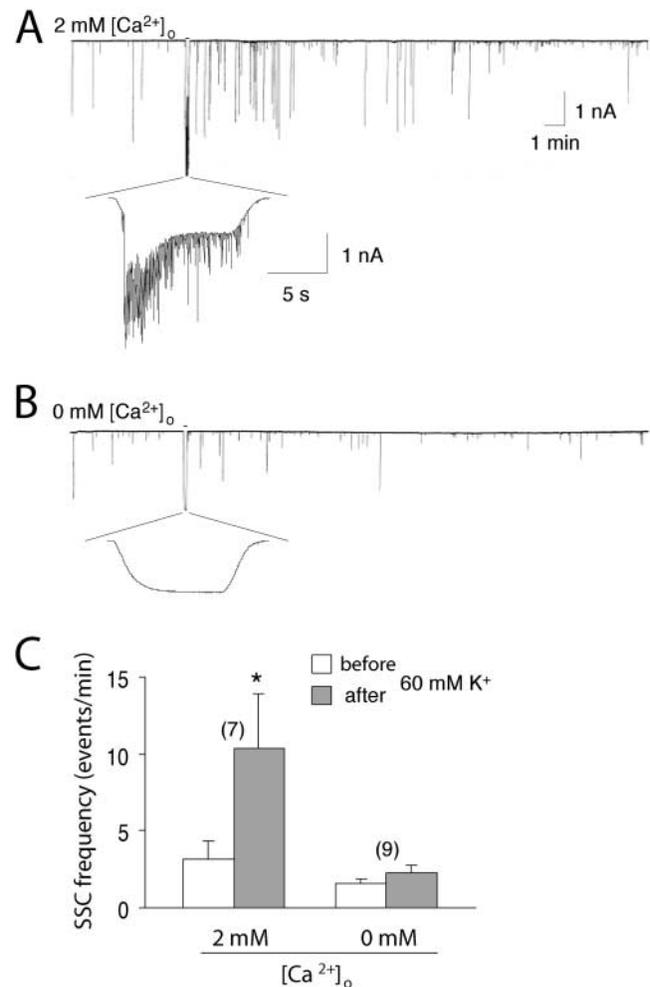
dependent  $\text{Na}^+$  channels would result in very little  $\text{Na}^+$  influx, providing further support that  $\text{Na}^+$  entry into the nerve terminals is critical for TISP. As summarized in Fig. 4 C, local perfusion of high  $\text{K}^+$  always elicited a significant increase in SSC frequency during depolarization in normal medium, but never did so in  $\text{Ca}^{2+}$ -free medium. As depolarization alone does not cause any increase in transmitter release in the absence of  $\text{Ca}^{2+}$  influx, it seems unlikely that TISP is mediated by direct depolarization of the nerve terminals.

We next tested whether a rise in intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) is required for TISP. Membrane-permeable EGTA (EGTA/AM) was used to buffer  $[\text{Ca}^{2+}]_i$ . The effectiveness of tetanus was markedly reduced when cells were pretreated with EGTA/AM for  $>30$ – $60$  min. The synaptic efficacy increased only threefold in 0.1 mM EGTA/AM, and the increase in SSC frequency was further attenuated when 1 mM EGTA/AM was used (Fig. 5 A). Similar effects were achieved when  $[\text{Ca}^{2+}]_i$  was buffered by another  $\text{Ca}^{2+}$



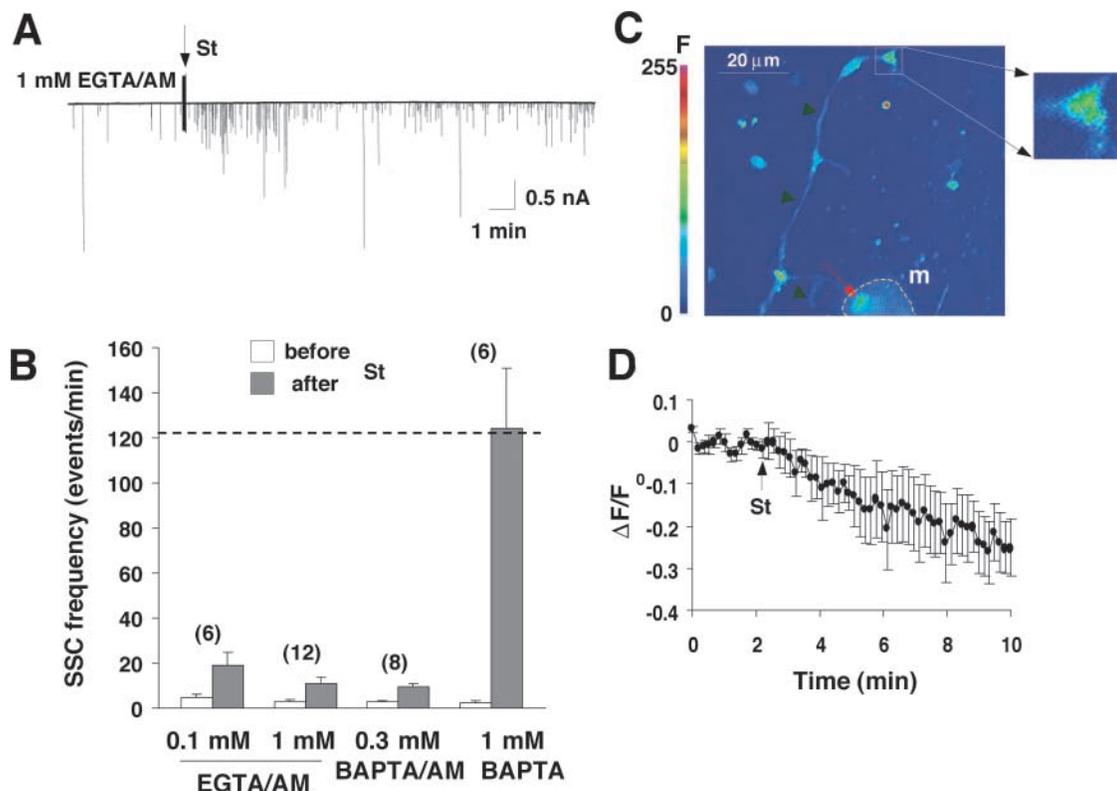
**Figure 3. Role of Na<sup>+</sup> influx in tetanus-induced potentiation.** (A) An example of TISP recorded in low extracellular Na<sup>+</sup> ( $[Na^+]_o = 57.5$  mM) and Ca<sup>2+</sup>-free conditions. To maintain the ionic strength of the low Na<sup>+</sup> solution, Na<sup>+</sup> ions were replaced by NMDG. (B) Comparison of TISP in normal and low Na<sup>+</sup> conditions. Note that the synaptic potentiation was much smaller in low  $[Na^+]_o$  than that in regular  $[Na^+]_o$  (115 mM). In this and all other figures, a *t* test was used for statistics unless indicated otherwise. \*\*\*,  $P < 0.0001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ . (C) Inhibition of TISP by local perfusion of TTX (0.5  $\mu$ M). Ringer solution containing TTX was perfused to the local area covering the synapse under recording, as indicated by the horizontal bar. Note that perfusion of TTX during tetanic stimulation completely blocked TISP. In contrast, the same tetanus induced potentiation subsequently in the same neurons when Ringer solution was perfused. (D) Summary of the effect of local TTX perfusion on TISP. A brief perfusion of TTX (0.5  $\mu$ M) completely prevented TISP, while similar perfusion with Ringer solution had no effect.

chelator, the membrane-permeable BAPTA/AM, for >30 min (Fig. 5 B). Interestingly, robust synaptic potentiation was induced by tetanus when the membrane-impermeable BAPTA was loaded directly into the postsynaptic muscle cells through the patch pipette (pipette concentration, 1 mM; Fig. 5 B). These results suggest that although independent of Ca<sup>2+</sup> influx, TISP still requires an increase in  $[Ca^{2+}]_i$  in the presynaptic neurons but not in the postsynaptic muscle cells. To determine whether the increase in SSC frequency truly reflects an enhanced transmitter release, and whether TISP is completely independent of postsynaptic muscle cells, we examined transmitter release at free nerve



**Figure 4. Depolarization of nerve terminals does not induce synaptic potentiation.** (A) Effect of local depolarization in normal Ca<sup>2+</sup> condition. After a period of control recording of SSCs, high K<sup>+</sup> solution (Ringer solution with 60 mM KCl) was applied (indicated by the bar) from a pipette brought near the synapse using the fast perfusion system to induce depolarization. High K<sup>+</sup> depolarized the nerve terminal as well as the muscle membrane, as reflected by a sustained downward shift of the baseline. The inset shows, at a higher time resolution, a dramatic increase in SSC frequency as a consequence of high K<sup>+</sup>-induced depolarization. (B) Effect of local depolarization in Ca<sup>2+</sup>-free medium. The experiment was done in the same way as in A except that Ca<sup>2+</sup> influx was completely blocked. Note that high K<sup>+</sup> induced depolarization of muscle membrane potential but there is no SSC at all (inset). (C) Summary of the effects of high K<sup>+</sup>-induced local depolarization in normal and Ca<sup>2+</sup>-free media.

terminals using FM dye destaining techniques (Betz et al., 1992; Ryan et al., 1993). FM 1-43 dye was loaded into synaptic vesicles in the presynaptic terminals by exposing neurons to high K<sup>+</sup> loading solution (60 mM K<sup>+</sup>, 2 mM Ca<sup>2+</sup>) for 3 min in the presence of FM 1-43 (2  $\mu$ M), followed by extensive washes in Ca<sup>2+</sup>-free medium. Fluorescent spots, which represent clusters of recycled vesicles labeled by FM dye, were quite stable in the Ca<sup>2+</sup>-free medium for a long period of time, suggesting that baseline SSC does not lead to significant FM dye destaining (Fig. 5 D). TISP was initiated by tetanic stimulation of the cell body of presynaptic neurons in the absence of extracellular Ca<sup>2+</sup>. A marked destaining of FM dye was observed at NMJ as well as free nerve



**Figure 5. Inhibition of TISP by lowering  $[Ca^{2+}]_i$ .** (A) A recording showing a reduced effect of tetanus in  $Ca^{2+}$ -free medium in the presence of EGTA/AM (1 mM). (B) The effects of  $Ca^{2+}$  chelators. Bath application of EGTA/AM (1 mM more effective than 0.1 mM) significantly reduced TISP. BAPTA/AM at 0.3 mM was as effective as EGTA/AM at 1 mM. 1 mM BAPTA, loading of membrane-impermeable BAPTA (1 mM) into postsynaptic muscle cells did not block TISP. Dashed line, TISP observed in control  $Ca^{2+}$ -free medium. (C) FM dye fluorescence image of an axon with a free nerve terminal and a terminal (indicated by the red arrow) that innervates a myocyte (m, highlighted by dashed line). The cell body of the spinal neuron is outside of the image. Green arrowheads indicate the axon of the neuron. Inset, an enlarged image of the free terminal. FM dye was loaded into the axon by a 3-min high  $K^+$  depolarization in the presence of FM 1-43 (2  $\mu M$ ) in normal medium, followed by extensive washes in  $Ca^{2+}$ -free medium. The pseudocolor scale shows approximate fluorescence intensity values in 8-bit scale. (D) Average destaining curve at free nerve terminals in  $Ca^{2+}$ -free medium. Arrow indicates the time point when tetanus was applied to initiate the destaining.  $n = 6$ .

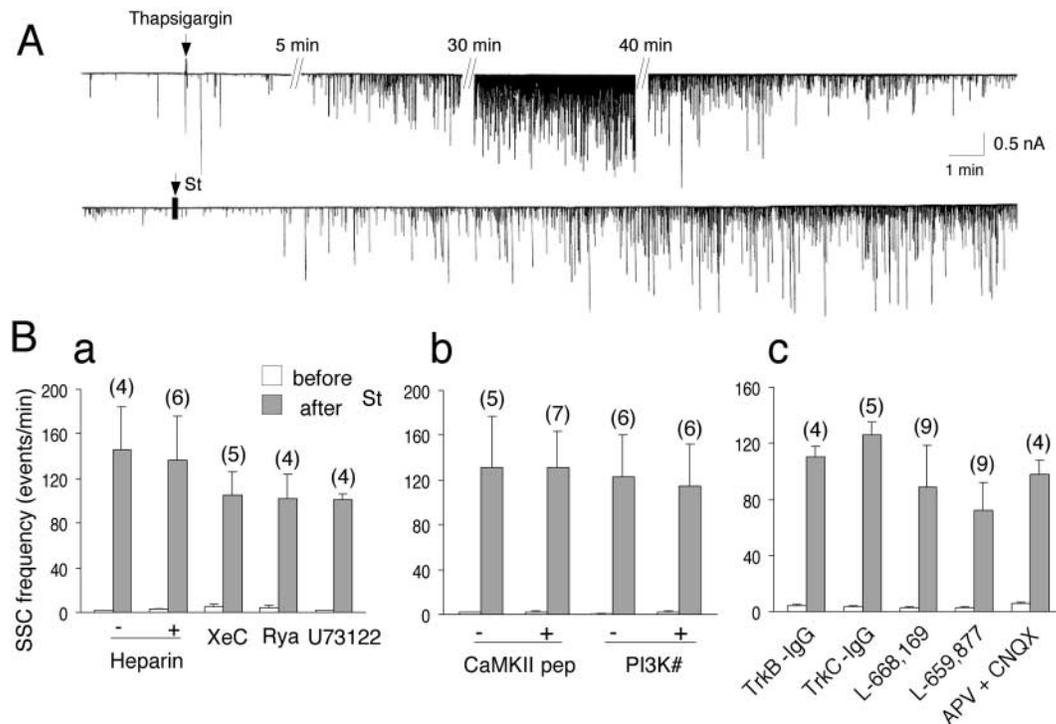
terminals (Fig. 5 D). Thus, the induction of TISP is completely independent of postsynaptic muscle cells.

### Search for mechanisms underlying TISP

In  $Ca^{2+}$ -free medium, ER  $Ca^{2+}$  stores are the major sources of intracellular  $Ca^{2+}$ . Two approaches were employed to investigate the role of  $Ca^{2+}$  from ER  $Ca^{2+}$  stores in TISP. First, we examined whether inhibition of  $Ca^{2+}$  release from the ER stores (the ryanodine receptor and the IP<sub>3</sub> receptor) would prevent TISP. A high concentration of ryanodine (Rya, 100  $\mu M$ ) may be used as a ryanodine receptor antagonist. If the TISP is mediated by opening of ryanodine receptor channels on the ER, pretreatment with ryanodine should prevent the effect of tetanus. However, TISP still occurred when the cultures were pretreated with ryanodine (Fig. 6 B, a). IP<sub>3</sub> is generated from phosphatidylinositol 4,5-bisphosphate (PI[4,5]P<sub>2</sub>) by PLC- $\gamma$  cleavage. Pretreatment of the nerve-muscle cocultures with the PLC- $\gamma$  inhibitor U73122 (5  $\mu M$ ) did not block TISP (Fig. 6 B, a). Application of the IP<sub>3</sub> receptor inhibitor Xestospongion C (XeC, 1  $\mu M$ ) (Gafni et al., 1997) also had no effect (Fig. 6 B, a). We also used heparin, a more specific and reliable inhibitor of the IP<sub>3</sub> receptor. As heparin is a hydrophilic molecule incapable of en-

tering cells through bath application, we loaded it into the presynaptic neurons through embryo injection techniques (Yang et al., 2001). The presence of heparin in presynaptic neurons was indicated by the fluorescence of coinjected rhodamine dextran. Tetanic stimulation induced very similar synaptic potentiation in both heparin-positive and -negative synapses (Fig. 6 B, a). In the second series of experiments, we determined whether TISP could still occur after ER  $Ca^{2+}$  stores are completely exhausted. Thapsigargin is known to inhibit ER  $Ca^{2+}$ -ATPase activity and therefore has frequently been used to deplete ER  $Ca^{2+}$  stores (Thastrup et al., 1990). With a few minutes of delay, the application of thapsigargin in  $Ca^{2+}$ -free conditions induced a marked increase in SSC frequency due to the release of  $Ca^{2+}$  from the ER  $Ca^{2+}$  stores. Within 60–80 min or so, the SSC frequency returned to control levels as a consequence of the depletion of ER  $Ca^{2+}$ . Tetanic stimulation at this point could still induce the synaptic potentiation (Fig. 6 A). Taken together, these results strongly indicate that the TISP was not mediated by the release of  $Ca^{2+}$  from the ryanodine or IP<sub>3</sub> receptor stores.

We further investigated the role of a number of key signaling molecules and extracellular factors implicated in the con-



**Figure 6. Characterization of signaling pathways.** (A) TISP occurs even after treatment with thapsigargin, which presumably depleted ER Ca<sup>2+</sup> stores. Note that application of thapsigargin (2  $\mu$ M) resulted in a gradual but massive increase in the SSC frequency due to the release of Ca<sup>2+</sup> from ER Ca<sup>2+</sup> stores. 1 h later, SSC frequency returned to its original levels, suggesting depletion of ER Ca<sup>2+</sup> stores. Application of tetanus at this point still elicited TISP. (B) Effects of inhibiting specific signal transduction pathways. The inhibition was achieved either by pretreatment with specific blockers (XeC; Rya, ryanodine; U73122; TrkB-IgG; etc.) or by loading inhibitors into presynaptic neurons (CaMKII peptide inhibitor, PI3K# mRNA, Heparin). (B, a) Role of PLC- $\gamma$ , IP<sub>3</sub> receptor, and ryanodine receptor. (B, b) Role of CaMKII and PI3 kinase. (B, c) Role of BDNF, NT3, substance P, and glutamate. None of these manipulations affected tetanus-induced potentiation.

trol of transmitter release. Ca<sup>2+</sup>/calmodulin-dependent kinase II (CaMKII) is well known for its ability to regulate transmitter release from nerve terminals (Greengard et al., 1993). Loading of a specific peptide inhibitor for CaMKII (Ishida et al., 1995) (estimated concentration, 10  $\mu$ M) into the presynaptic neurons by embryo injection had no effect on TISP (Fig. 6 B, b). PI3K has recently been implicated in the modulation of transmitter release (Yang et al., 2001). However, expression of a dominant-negative form of PI3K (PI3K#) in presynaptic neurons by injecting PI3K# mRNA into the *Xenopus* embryos did not interfere with synaptic potentiation induced by tetanus (Fig. 6 B, b). A number of secretory factors, such as neurotrophins, glutamate, and substance P, have been shown to potentiate synaptic transmission at NMJ (Lohof et al., 1993; Fu et al., 1995), and the release of some of these factors could be independent of Ca<sup>2+</sup> influx (Canossa et al., 2001). We tested whether tetanic stimulation induces the release of these factors, leading to the synaptic potentiation. Pretreatment of the cultures with TrkB-IgG (4  $\mu$ g/ml) or TrkC-IgG (10  $\mu$ g/ml), scavenger molecules specific for the neurotrophins BDNF and NT3, respectively, had no effect on the TISP (Fig. 6 B, c). Pretreatment of the cultures with a cocktail of glutamate receptor antagonists (CNQX, 5  $\mu$ M, + Apv, 50  $\mu$ M) also failed to block the effect of tetanus (Fig. 6 B, c). Finally, inhibition of the substance P receptors NK1 and NK2 by L-668,169 (6  $\mu$ M) and L-659,877 (6  $\mu$ M) did not prevent the synaptic

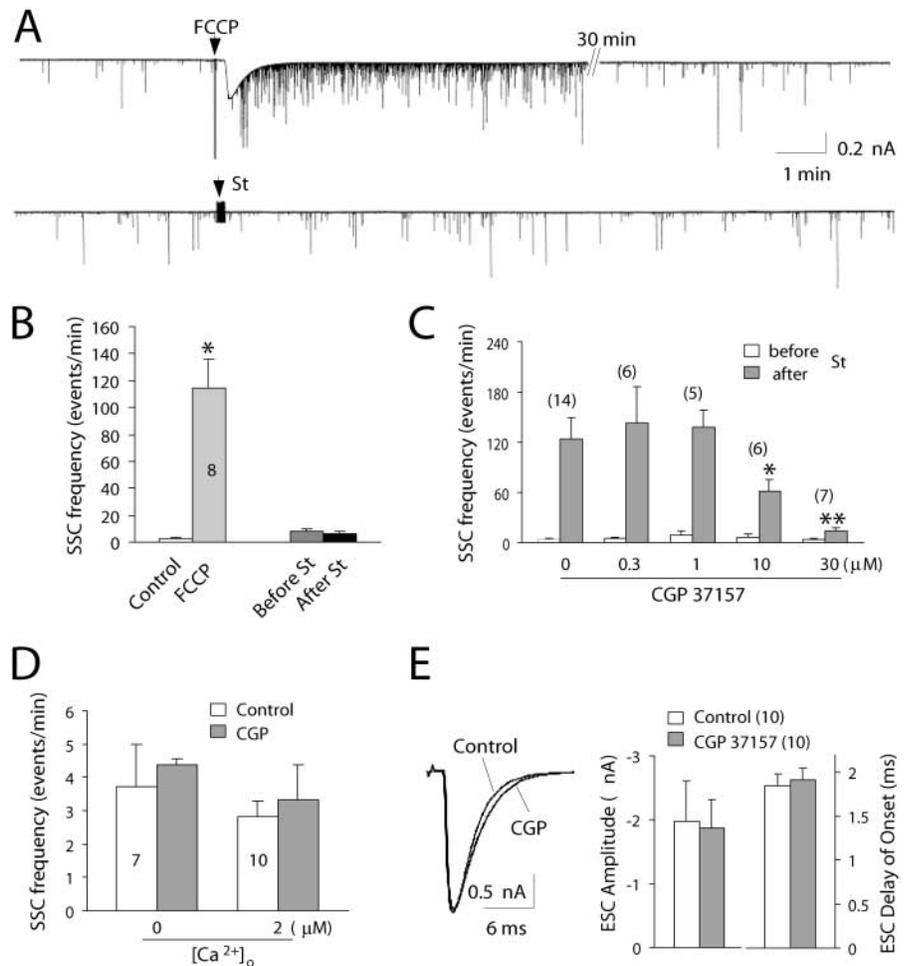
potentiation (Fig. 6 B, c). Thus, it seems unlikely that tetanus-induced release of the secretory factors from presynaptic neurons could explain the synaptic potentiation.

#### Requirement for mitochondrial Na<sup>+</sup>-Ca<sup>2+</sup> exchanger

Tetanic stimulation rapidly increases the concentration of intracellular Na<sup>+</sup>, which triggers the exchange of cytoplasmic Na<sup>+</sup> with Ca<sup>2+</sup> in the mitochondria (Friel, 2000; Gunter et al., 2000). The requirement of intracellular Ca<sup>2+</sup> as well as Na<sup>+</sup> influx, and the independence of Ca<sup>2+</sup> influx and ER Ca<sup>2+</sup> stores, suggests that the TISP is mediated by mitochondrial Ca<sup>2+</sup>. To test this hypothesis, we first uncoupled mitochondrial membrane potential by the protonophore carbonyl cyanide 4-(trifluoromethoxy) phenyl hydrazone (FCCP, 1  $\mu$ M), which depolarizes and depletes Ca<sup>2+</sup> from mitochondria. Application of FCCP in Ca<sup>2+</sup>-free conditions induced a huge increase in SSC frequency, which returned to control levels in 20–50 min (Fig. 7, A and B). FCCP also caused a transient and slow drift of membrane potential, which was recovered within minutes (Fig. 7 A). Compared with the effect of thapsigargin on ER Ca<sup>2+</sup> stores, the depletion of mitochondria Ca<sup>2+</sup> by FCCP had a faster onset and higher magnitude, but lasted for a relatively shorter period of time. Importantly, tetanic stimulation 40 min after FCCP application could no longer induce synaptic potentiation (Fig. 7 A). Quantitative analysis indicated that FCCP treatment elicited a transient, 67-fold increase in SSC

**Figure 7. Role of the mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger in tetanus-induced potentiation.**

(A) Effect of the mitochondria decoupler FCCP on SSCs. Application of FCCP (arrow) elicited a transient but marked increase in SSC frequency due to the  $\text{Ca}^{2+}$  release from mitochondria. (B) Effect of the mitochondria decoupler FCCP on SSCs. 1 h after the application of FCCP, the SSC frequency returned to normal. Tetanic stimulation could no longer increase the SSC frequency.  $n = 8$ . (C) Dose-dependent inhibition of TISP by CGP. Cells were pretreated with different concentrations of CGP, a specific inhibitor for the mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger, before application of tetanus. The reduction in TISP was significant when CGP reached  $10 \mu\text{M}$  and  $30 \mu\text{M}$  (ANOVA followed by post hoc tests; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). (D) CGP ( $30 \mu\text{M}$ ) does not affect the basic properties of SSCs in both normal and  $\text{Ca}^{2+}$ -free media before tetanic stimulation. (E, left) Superimposed traces of ESCs before and after CGP treatment. (E, right) Summary of CGP effects on ESC amplitude and delay of onset.

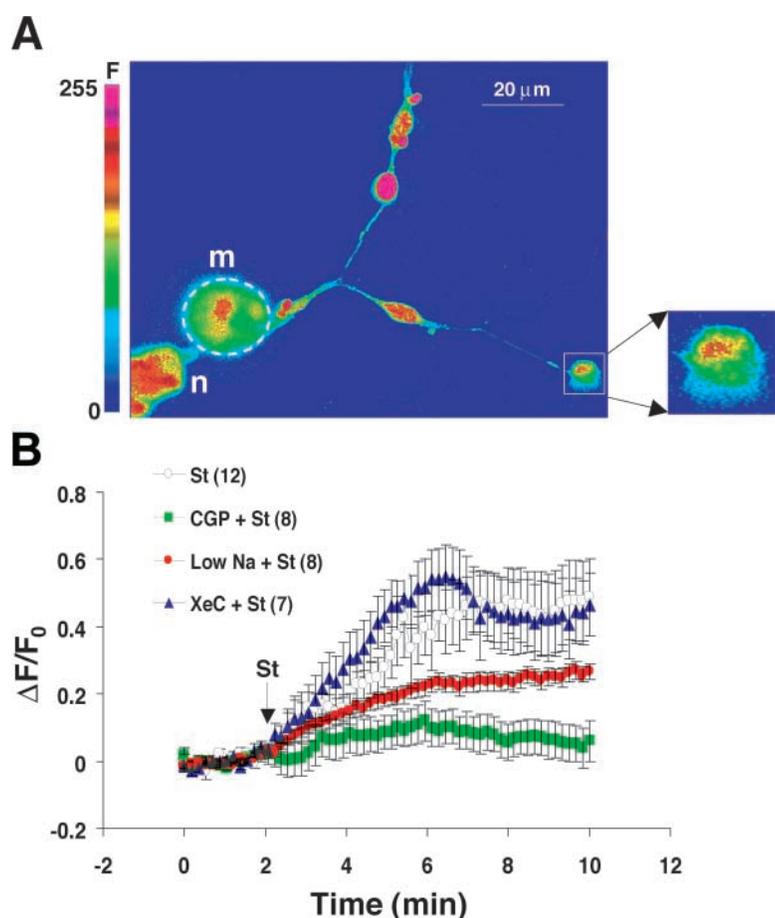


frequency, and TISP failed to occur when the tetanic stimulation was applied after the effect of FCCP subsided (Fig. 7 B). These data suggest that the release of  $\text{Ca}^{2+}$  from mitochondria is critical for TISP.

To determine the specific route through which  $\text{Ca}^{2+}$  came out of mitochondria, we used CGP37157 (CGP), a specific inhibitor for the mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger (Cox et al., 1993). Pretreatment of cells with CGP markedly reduced the effectiveness of tetanus to induce synaptic potentiation. Dose-response experiments showed that CGP was effective at as low as  $10 \mu\text{M}$  (Fig. 7 C). Quantitative analysis indicated that the SSC frequency increased 68-fold after tetanus in control conditions, but only 3.5-fold at the NMJ treated with  $30 \mu\text{M}$  of CGP (Fig. 7 C). To ensure the specificity of CGP, we performed a number of control experiments. In either normal or  $\text{Ca}^{2+}$ -free conditions, CGP ( $30 \mu\text{M}$ ) did not affect the frequency or any other parameters of SSCs (Fig. 7 D and not depicted), suggesting that it does not alter the release apparatus. Treatment of the neuromuscular synapses with CGP at the same concentration had no effect on the properties of ESCs induced in normal  $\text{Ca}^{2+}$  conditions (Fig. 7 E). Identical action potentials were recorded in spinal neurons treated with or without CGP (not depicted). Thus, TISP appears to require  $\text{Ca}^{2+}$  release from the mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger.

We next examined whether tetanic stimulation elicits an increase in  $[\text{Ca}^{2+}]_i$  via the mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  ex-

changer.  $\text{Ca}^{2+}$  imaging was performed by confocal microscopy using the cell-permeable  $\text{Ca}^{2+}$  indicator fluo-4. Cells in the nerve-muscle cultures were loaded with this indicator, and tetanic stimulation was applied to neuronal soma through a loose-patch pipette (touched, but not sealed) to induce action potentials. We determined the fluo-4 fluorescence in  $\text{Ca}^{2+}$ -free medium (Fig. 8 A, inset) and normalized it to averaged basal fluorescence before stimulation. The change in fluorescence ( $\Delta F/F_0$ ) was plotted against time. Tetanic stimulation evoked a gradual increase of  $[\text{Ca}^{2+}]_i$  at nerve terminals (Fig. 8 B), with the time course very similar to that of TISP. This increase was significantly attenuated in low  $[\text{Na}^+]_o$  ( $57.5 \text{ mM}$ , Fig. 8 B). Moreover, pretreatment with CGP markedly attenuates the tetanus-induced mitochondrial  $\text{Ca}^{2+}$  release (Fig. 8 B). The basal  $[\text{Ca}^{2+}]_i$  level in the terminals, however, was not affected by CGP (see Fig. 10 C). In the presence of the IP<sub>3</sub> receptor inhibitor XeC, tetanus still induced the same magnitude of increase in  $[\text{Ca}^{2+}]_i$  (Fig. 8 B). Thus, in zero  $[\text{Ca}^{2+}]_o$  conditions, tetanus-induced increase in intracellular  $\text{Ca}^{2+}$  is mediated by the mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger. These results, together with the findings that TISP could be attenuated by FCCP and CGP, strongly support the model that tetanic stimulation induces  $\text{Ca}^{2+}$  release through the mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger at the nerve terminals, leading to a marked potentiation of transmitter release.



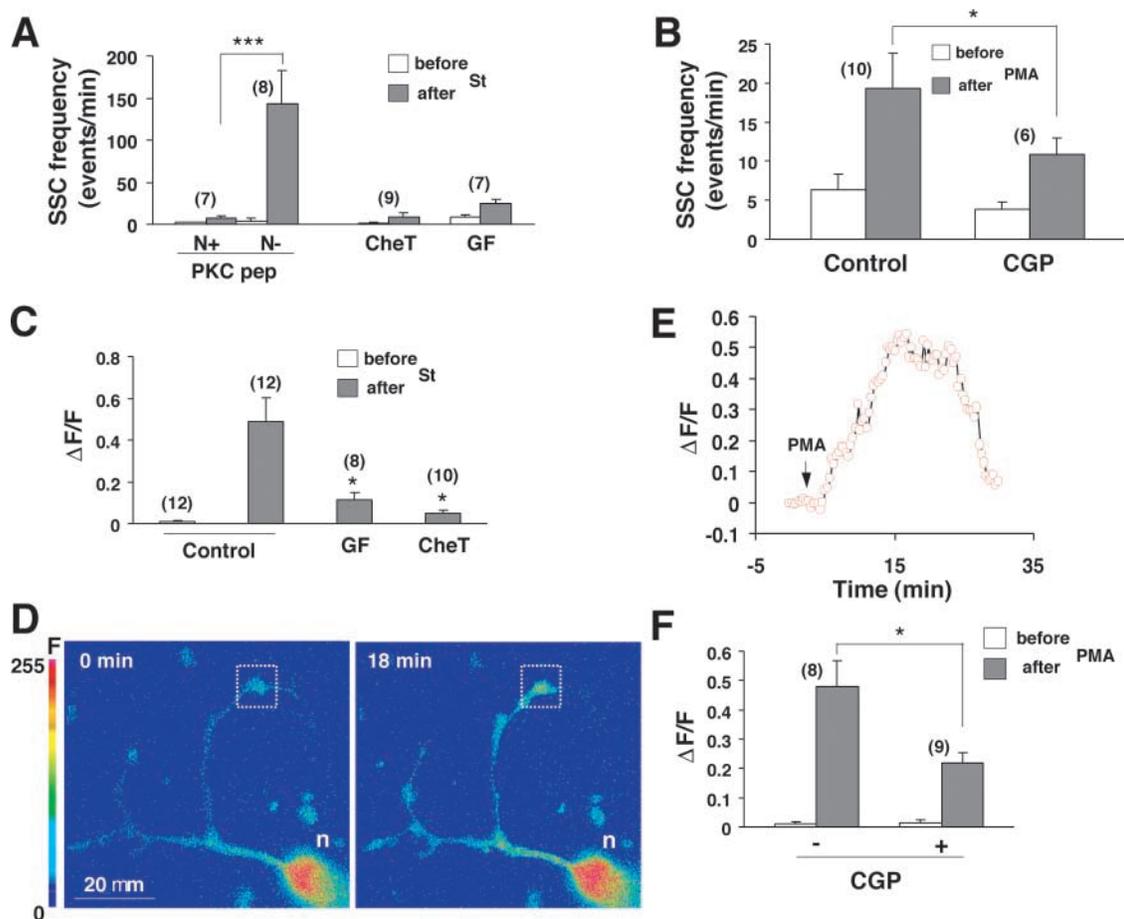
**Figure 8. Tetanus-induced Ca<sup>2+</sup> release from mitochondria in Ca<sup>2+</sup>-free medium.** Cytosolic Ca<sup>2+</sup> was measured using fluo-4 as an indicator. (A) An image of fluo-4-filled spinal neuron (n) and myocyte (m). The pseudocolor scale shows approximate fluorescence intensity values in 8-bit scale. The main axon of the neuron innervates a myocyte (highlighted by dashed line) and then bifurcates to form two axonal branches. (A, inset) An amplified image of a free nerve terminal. (B) Time course of tetanus-induced changes in fluo-4 fluorescence. The images were measured using region-of-interest tool outlining varicosities and were post hoc normalized to initial fluorescence ( $\Delta F/F_0$ ). The numbers after the legend indicate the number of experiments performed.

### Regulation of TISP by PKC and physiological role of TISP in normal extracellular Ca<sup>2+</sup>

While many intracellular signaling pathways and secretory factors we tested had no effect on TISP (Fig. 6), inhibition of PKC had a profound effect on TISP as well as tetanus-induced [Ca<sup>2+</sup>]<sub>i</sub> increase. Loading of a specific, peptide inhibitor for PKC (PKC pep; House and Kemp, 1987) into the presynaptic neurons by embryo injection dramatically reduced the potentiation induced by tetanic stimulation of the same neurons (N+), while TISP was observed in the nearby synapses made by neurons not loaded with the PKC pep (N-) in the same culture dishes (Fig. 9 A). Again, loading of the PKC pep into the postsynaptic muscle cells had no effect on TISP (unpublished data). In addition, TISP was greatly attenuated by pretreatment with the specific PKC inhibitor chelerythrine (CheT, 5 μM) or GF 1092303X (GF, 0.8 μM), respectively (Fig. 9 A). On the other hand, application of the PKC activator PMA (5 μM) alone was capable of potentiating transmitter release in the absence of Ca<sup>2+</sup> influx, although the magnitude of the potentiation was smaller than that of TISP, and pretreatment with CGP significantly reduced the PMA-induced potentiation (Fig. 9 B). We then investigated the role of PKC in intracellular Ca<sup>2+</sup>. Inhibition of endogenous PKC by CheT or GF significantly reduced the tetanus-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 9 C). On the other hand, activation of PKC by PMA rapidly and significantly increased the [Ca<sup>2+</sup>]<sub>i</sub> in Ca<sup>2+</sup>-free medium (Fig. 9 D). The time course of the [Ca<sup>2+</sup>]<sub>i</sub> rise was similar to that induced by tetanus, but the level of [Ca<sup>2+</sup>]<sub>i</sub>

gradually declined even in the presence of PMA (Fig. 9 E). Further, the PMA-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> was inhibited by CGP (Fig. 9 F). Thus, while PKC is required for TISP and the tetanus-induced increase in [Ca<sup>2+</sup>]<sub>i</sub>, activation of PKC is not sufficient to induce the same magnitude of synaptic potentiation as TISP, or the same patterns of [Ca<sup>2+</sup>]<sub>i</sub> change as that induced by tetanus. These results suggest that PKC serves as a modulator, rather than a mediator, for tetanus-induced [Ca<sup>2+</sup>]<sub>i</sub> release through the mitochondrial Na<sup>+</sup>-Ca<sup>2+</sup> exchanger.

Finally, we determined whether the mitochondrial Na<sup>+</sup>-Ca<sup>2+</sup> exchanger also contributes to the synaptic plasticity in normal extracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>o</sub> = 2 mM). The synaptic potentiation induced by the same tetanus (50 Hz, 10 s) in normal medium was even greater than that in Ca<sup>2+</sup>-free medium (Fig. 10 A). Pretreatment with CGP greatly attenuated the effect of the tetanus (Fig. 10 A). The SSC frequency increased 143-fold after tetanus, but only 38-fold at the NMJ treated with 30 μM CGP. To detect high-amplitude, rapid changes of nerve terminal [Ca<sup>2+</sup>]<sub>i</sub> in normal [Ca<sup>2+</sup>]<sub>o</sub> conditions, we used a low affinity Ca<sup>2+</sup> indicator Oregon green 488 BAPTA-5N, AM (OG488, 5 μM). Consistent with a previous report (David et al., 1998), a train of tetanic stimulation induced a rapid increase in [Ca<sup>2+</sup>]<sub>i</sub> due to Ca<sup>2+</sup> influx into the cytosol, followed by a slow but sustained increase in [Ca<sup>2+</sup>]<sub>i</sub>, most likely due to Ca<sup>2+</sup> release from mitochondria (Fig. 10 B). Pretreatment with CGP blocked the late onset [Ca<sup>2+</sup>]<sub>i</sub> increase (Fig. 10 B), but not the increase of [Ca<sup>2+</sup>]<sub>i</sub> immediately after the tetanus (Fig. 10 B, inset). Application



**Figure 9. Role of PKC in tetanus-induced potentiation and mitochondrial  $\text{Ca}^{2+}$  release.** (A) Inhibition of PKC blocks TISP. PKC was inhibited either by loading a specific peptide inhibitor into presynaptic neurons through embryo injection or by bath application of the PKC inhibitor CheT or GF. (B) Activation of PKC enhances transmission by inducing mitochondria  $\text{Ca}^{2+}$  release. Synaptic activities were recorded before and after PMA application. Note that inhibition of mitochondria  $\text{Ca}^{2+}$  release by CGP significantly reduced the effect of PMA. (C) Quantification of tetanus-induced increase in  $[\text{Ca}^{2+}]_i$  rise and its inhibition by CheT or GF. The data were normalized to initial value  $F_0$  and expressed as  $\Delta F/F_0$ . \*, significantly lower compared with "St" alone.  $P < 0.05$ , ANOVA followed by post hoc tests. (D) Fluo-4 images of a neuron right before (left) and 18 min after (right) application of PMA. (E) A representative time course of changes in fluo-4 fluorescence induced by bath application of PMA. (F) Quantification of PMA-induced increase in  $[\text{Ca}^{2+}]_i$  rise and its inhibition by CGP.

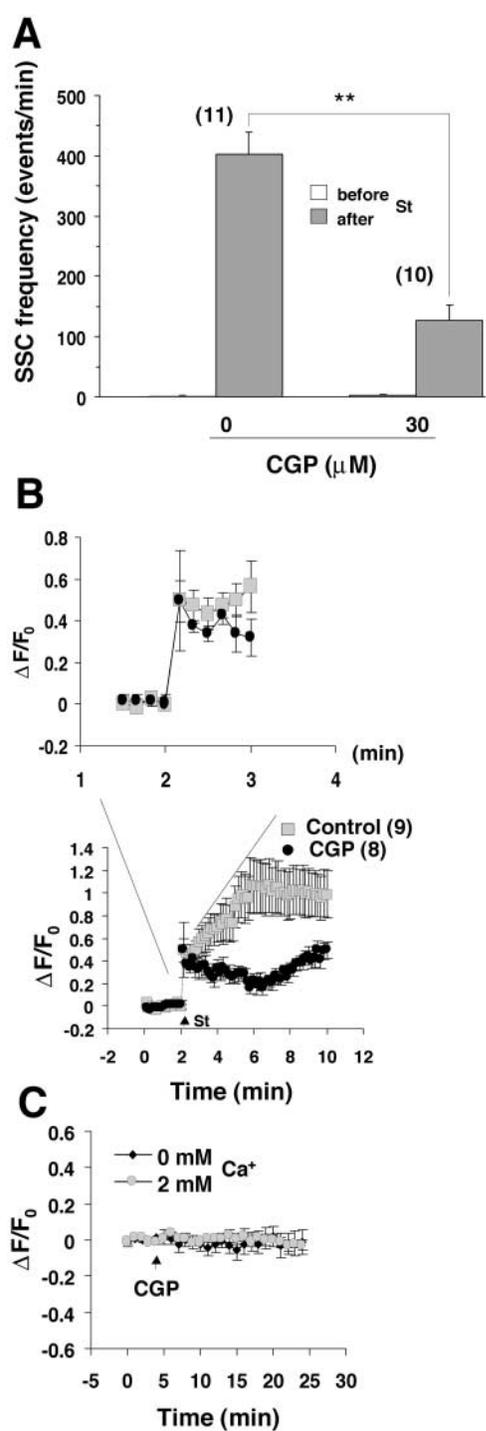
of CGP did not affect the basal level of  $[\text{Ca}^{2+}]_i$  in either normal or  $\text{Ca}^{2+}$ -free medium (Fig. 10 C). Taken together, these results suggest that the mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger contributes to TISP under normal physiological conditions.

## Discussion

The mechanism underlying modulation of transmitter release is an important but difficult problem to study, because many extracellular and intracellular molecules that regulate transmitter release also affect  $\text{Ca}^{2+}$  influx. Thus, it is difficult to establish whether an effect on synaptic transmission is due to modulation of  $\text{Ca}^{2+}$  influx or to direct regulation of transmitter release. The present study was done in the complete absence of  $\text{Ca}^{2+}$  influx, avoiding the potential problem of channel modulation. Using this approach, we have discovered a novel form of synaptic plasticity in which transmitter release is dramatically potentiated in response to a presynaptic tetanic stimulation. This form of synaptic plasticity would not have been observed if the experiments were performed in normal extracellular  $\text{Ca}^{2+}$ . There are a number of

interesting findings: (1)  $\text{Na}^+$  influx appears to be the trigger for TISP; (2) it requires an increase in  $[\text{Ca}^{2+}]_i$ ; (3) the increase in  $[\text{Ca}^{2+}]_i$  results from  $\text{Ca}^{2+}$  efflux through the mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger; and (4) PKC is involved in TISP as well as mitochondrial  $\text{Ca}^{2+}$  efflux. The enhancement of transmitter release via  $\text{Ca}^{2+}$  efflux from mitochondria also occurs in normal  $[\text{Ca}^{2+}]_o$ , and may contribute to synaptic modulation in heightened activity under physiological conditions such as physical exercise, and/or in pathological conditions such as neuromuscular fatigue. These results demonstrate the role of  $\text{Na}^+$  influx and the mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger in synaptic modulation and reveal a new form of synaptic plasticity. Further, our study may help understand how mitochondria and PKC contribute to the regulation of transmitter release and provide a useful model to investigate molecular mechanisms for transmitter release without the interference of  $\text{Ca}^{2+}$  influx.

Although our study demonstrated that transmission and synaptic plasticity could occur without  $\text{Ca}^{2+}$  influx, it seems that a rise of presynaptic  $[\text{Ca}^{2+}]_i$  is still necessary. This is quite different from the  $\text{Ca}^{2+}$ -independent vesicle fusion in



**Figure 10. Role of the mitochondrial Na<sup>+</sup>-Ca<sup>2+</sup> exchanger in normal extracellular Ca<sup>2+</sup> conditions.** Cultures were treated with CGP for 30 min before application of tetanus. (A) Inhibition of TISP by CGP in medium containing normal [Ca<sup>2+</sup>]<sub>o</sub>. (B) Inhibition of tetanus-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> by CGP in medium containing normal [Ca<sup>2+</sup>]<sub>o</sub>. Cytosolic Ca<sup>2+</sup> was measured using OG488 as an indicator. Note that CGP had no effect on the initial increase of [Ca<sup>2+</sup>]<sub>i</sub> immediately after the tetanus, but suppressed the late rise of [Ca<sup>2+</sup>]<sub>i</sub>. (B, bottom) Initial rates of [Ca<sup>2+</sup>]<sub>i</sub> increase in control and CGP-treated spinal terminals on an expanded time scale. (C) Effect of CGP on basal level of [Ca<sup>2+</sup>]<sub>i</sub> in normal and Ca<sup>2+</sup>-free medium. Arrow indicates the application of CGP (final concentration, 30 mM).

the cell body of DRG neurons reported recently (Zhang and Zhou, 2002). Vesicle fusion, as measured by membrane capacitance recording, was induced by membrane depolarization in Ca<sup>2+</sup>-free medium. Fura-2 measurements could not detect any change in [Ca<sup>2+</sup>]<sub>i</sub> under these conditions. Even intracellular dialysis with BAPTA for 10 min could not block this secretion. It was therefore concluded that a Ca<sup>2+</sup>-independent but voltage-dependent vesicular secretion may exist in the cell body of the DRG neurons (Zhang and Zhou, 2002). As the authors pointed out, the depolarization-induced vesicle fusion was not observed in a number of other cells tested. In our system, depolarization of the nerve terminals in Ca<sup>2+</sup>-free conditions by rapid perfusion of high K<sup>+</sup> solution could not induce transmitter release at the neuromuscular synapses. Although the present study also found an increase in vesicle fusion in the absence of Ca<sup>2+</sup> influx, we attribute this phenomenon to Na<sup>+</sup> influx driven by action potentials and Ca<sup>2+</sup> release through mitochondrial Na<sup>+</sup>-Ca<sup>2+</sup> exchanger. It will be interesting to determine whether mitochondrial Ca<sup>2+</sup> release also contributes to the depolarization-induced vesicle fusion seen in DRG cell bodies.

Mitochondria are observed in virtually all nerve terminals. It has long been thought that the function of the terminal mitochondria is to provide energy necessary for synaptic vesicle cycling. However, a number of studies have demonstrated that high frequency stimulation could induce mitochondrial Ca<sup>2+</sup> release at the motoneuron terminals, and this could go on over a period of ~10 min after the cessation of the stimulation (David et al., 1998; David, 1999). The release of mitochondrial Ca<sup>2+</sup> has been shown to enhance transmitter release induced by repetitive stimulation or drug application (Tang and Zucker, 1997; Scotti et al., 1999; Tsang et al., 2000). As these experiments were done in normal extracellular Ca<sup>2+</sup>, loading of Ca<sup>2+</sup> into the mitochondria during stimulation and unloading after stimulation is thought to be the mechanism for the transient increase in [Ca<sup>2+</sup>]<sub>i</sub> (Tang and Zucker, 1997; Melamed-Book and Rahamimoff, 1998). Mitochondria are therefore proposed to serve as a Ca<sup>2+</sup> buffering system that controls the extent of cytosolic Ca<sup>2+</sup> rise in the nerve terminals (Stuenkel, 1994; Tang and Zucker, 1997; David et al., 1998; David, 1999). We found, however, that FCCP could induce mitochondrial Ca<sup>2+</sup> release when Ca<sup>2+</sup> influx was completely prohibited. Thus, resting mitochondria contain substantial amounts of Ca<sup>2+</sup>, and the release of this Ca<sup>2+</sup> is sufficient to enhance transmitter release at NMJ. Another advance by the present study is to demonstrate synaptic modulation by Ca<sup>2+</sup> release through the mitochondrial Na<sup>+</sup>-Ca<sup>2+</sup> exchanger. Several previous studies have suggested that the plasmalemmal Na<sup>+</sup>-Ca<sup>2+</sup> exchanger, but not the one on mitochondria, is important for presynaptic Ca<sup>2+</sup> regulation (Scotti et al., 1999; Zhong et al., 2001). We show that in Ca<sup>2+</sup>-free medium, inhibition of the mitochondrial Na<sup>+</sup>-Ca<sup>2+</sup> exchanger prevented the tetanus-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> and greatly attenuated TISP. Thus, our study reveals a previously unexpected role of the mitochondrial Na<sup>+</sup>-Ca<sup>2+</sup> exchanger in modulation of transmitter release.

A form of plasticity most relevant to the present study is PTP. Using the crayfish NMJ as a model system, Tang and Zucker (1997) have investigated the mechanism for PTP in

detail. Inhibition of mitochondrial  $\text{Ca}^{2+}$  uptake or release by tetraphenylphosphonium ( $\text{TPP}^+$ ), carbonyl cyanide m-chlorophenylhydrazone (CCCP), or ruthenium red all block PTP and the “residual  $\text{Ca}^{2+}$ ” in the nerve terminals after tetanic stimulation. They proposed that mitochondria accumulate  $\text{Ca}^{2+}$  during tetanic stimulation (as a consequence of  $\text{Ca}^{2+}$  influx) and release  $\text{Ca}^{2+}$  back to the cytoplasm after the tetanus. In a later study, they found that the specific plasmalemmal  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger inhibitor KB R7943 significantly reduced PTP as well as  $\text{Ca}^{2+}$  accumulation caused by  $\text{Na}^+$  influx (Zhong et al., 2001). In contrast, the specific mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger inhibitor CGP had no effect on either PTP or  $\text{Ca}^{2+}$  accumulation. It was therefore concluded that the plasmalemmal  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger acting in reverse mode is the key mediator for PTP, and the mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger is not involved in this form of plasticity. The TISP described in the present study is very different from PTP. First, TISP does not involve residual  $\text{Ca}^{2+}$  resulting from tetanus-induced  $\text{Ca}^{2+}$  influx. Rather, it can be induced in the complete absence of  $\text{Ca}^{2+}$  influx. Second, the kinetics of TISP is quite different from that of PTP. PTP occurs right after the termination of tetanus and gradually decreases over time. In  $\text{Ca}^{2+}$ -free medium, TISP occurs several minutes after the termination of the tetanus, peaks around 10 min, and lasts for a much longer period of time ( $>1$  h). Third, TISP and PTP are mediated by very different mechanisms. We demonstrated that TISP requires the mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger, while Zhong et al. (2001) showed that PTP is completely independent of the molecule. Taken together, the present study has identified a new form of synaptic plasticity and elucidated its underlying mechanisms.

Tetanic stimulation has also recently been shown to induce LTP at the NMJ (Wan and Poo, 1999). Injection of BAPTA, EGTA, or a peptide inhibitor for the  $\text{Ca}^{2+}$ -dependent phosphatase calcineurin into postsynaptic muscle cells blocks this LTP. Thus, the induction of LTP requires  $\text{Ca}^{2+}$  influx and perhaps calcineurin activity in postsynaptic muscle cells. We found that TISP in the absence of  $\text{Ca}^{2+}$  influx could also last for a long period of time (for  $>1$  h, or as long as the recording could be held, SSC frequency never returned to the baseline levels before tetanus). However, introduction of BAPTA or PKC inhibitor into postsynaptic muscle cells could not prevent TISP. FM dye destaining experiments also indicate that tetanic stimulation of neuronal soma elicited a robust transmitter release in free nerve terminals. Thus, TISP described in this study is independent of postsynaptic muscle cells.

At a variety of synapses, activation of PKC potentiates transmitter release primarily in steps downstream of  $\text{Ca}^{2+}$  influx (Majewski and Iannazzo, 1998). Given the same magnitude of  $[\text{Ca}^{2+}]_i$  rise, two factors control the fusion of synaptic vesicles and the release of transmitters: the number of readily releasable vesicles (also called the readily releasable pool) and the release probability of these vesicles. Using chromaffin cells and cultured hippocampal neurons as model systems, PKC has been implicated in the potentiation of synaptic transmission by increasing the size of the readily releasable pool (Gillis et al., 1996; Stevens and Sullivan, 1998). At calyx-type synapses in the brain stem, PKC is thought to facilitate vesicle exocytosis by increasing the probability of release

(Yawo, 1999; Wu and Wu, 2001). These conclusions, however, were based on experiments using phorbol ester as a PKC activator. A recent study indicated that phorbol ester-induced potentiation of transmitter release is mediated not by PKC but by Munc-13, a protein localized in the presynaptic active zone and involved in priming vesicles to fusion competence (Rhee et al., 2002). PKC may also modulate voltage-gated ion channels, leading to an increase in presynaptic  $\text{Ca}^{2+}$  influx (Fu and Huang, 1994; Byrne and Kandel, 1996). By eliminating  $\text{Ca}^{2+}$  influx into the nerve terminals, we were able to address the role of PKC in transmitter release induced by  $\text{Ca}^{2+}$  released from mitochondria. Inhibition of presynaptic PKC by several specific PKC inhibitors markedly attenuated the tetanus-induced  $\text{Ca}^{2+}$  release from mitochondria and virtually prevented TISP, while activation of PKC rapidly increased the  $[\text{Ca}^{2+}]_i$  and potentiated transmitter release in  $\text{Ca}^{2+}$ -free medium. Our results therefore support the notion that PKC is required for optimal function of the mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger at nerve terminals.

## Materials and methods

### Embryo injection, cell culture, and electrophysiology

As previously described (Yang et al., 2001), dominant-negative PI3 kinase mRNA, IP3 receptor inhibitor heparin (Sigma-Aldrich), PKC peptide inhibitor, or CaMKII peptide inhibitor (both from Calbiochem) was mixed with GFP mRNA and injected (total 10 ng) into one of the cells of two-cell stage *Xenopus* embryos. At stage 20–22, the normal or injected embryos were dissociated in  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$ -free medium, plated on glass coverslips, and grown for 1 d at room temperature (20–22°C). Synaptic currents were recorded from innervated muscle cells by whole-cell recording in either normal or  $\text{Ca}^{2+}$ -free medium. In the low  $[\text{Na}^+]_o$  experiments, the extracellular  $\text{Na}^+$  was reduced by half, plus 57.5 mM NMDG to maintain the ionic strength (Sigma-Aldrich). A time course of SSC frequency was first constructed on a minute-to-minute basis for each synapse. The SSC frequencies recorded 10 min before tetanic stimulation and those recorded during a 10-min period with the maximal response after tetanic stimulation were compared. Tetanic stimulation was applied to neuronal soma under loose seal conditions through a patch electrode filled with Ringer solution. For local perfusion of TTX to the NMJ, whole culture dishes were continuously perfused with  $\text{Ca}^{2+}$ -free or  $\text{Ca}^{2+}$ -containing Ringer solution. A TTX-containing pipette was positioned near synapses. After a period of control recording of SSCs, TTX (0.5  $\mu\text{M}$  in Ringer solution) was rapidly perfused to the synapse using a multi-barrel perfusion system (Warner Instruments). For high  $\text{K}^+$ -mediated depolarization experiments, the same system was used to perfuse high  $\text{K}^+$  solution (Ringer solution with 60 mM KCl) to the synapses. The duration and the extent of depolarization were reflected by the sustained downward shift of baseline. The clusters of synaptic currents were quite reproducible among different depolarization episodes.

### FM 1-43 imaging

The FM dye experiments were performed essentially as previously described (Wang et al., 2001). FM 1-43 (Molecular Probes) was loaded into the spinal neurons by incubating 1-d-old cultures with high  $\text{K}^+$  loading solution containing KCl (60 mM), NaCl (57.6 mM),  $\text{CaCl}_2$  (2 mM), Hepes (10 mM, pH 7.6), and FM 1-43 (2  $\mu\text{M}$ ) for 3 min. Cells were then rinsed with  $\text{Ca}^{2+}$ -free medium and imaged with a Noran Odyssey II confocal unit, using a laser with a band pass excitation filter around 488 nm and a 515-nm long pass emission filter. Four  $640 \times 480$  pixel frames were averaged (123 ms), and images were acquired at one image/10 s. The fluorescence images were stable with minimum bleach for  $>10$  min in  $\text{Ca}^{2+}$ -free medium. After acquiring 12 images (2 min) as baseline, FM 1-43 destaining was initiated by tetanus, and acquisition was continued for another 8 min. Fluorescence intensity was measured using a region-of-interest tool outlining the varicosities, corrected for photo bleaching, and post hoc normalized to initial fluorescence ( $\Delta F/F_0$ ).

### $\text{Ca}^{2+}$ imaging

Cells from 1-d-old cultures were loaded with  $\text{Ca}^{2+}$  indicators for 30–60 min. Fluo-4 (Molecular Probes; final concentration, 5  $\mu\text{M}$ ) was used for

terminal [Ca<sup>2+</sup>]<sub>i</sub> changes in Ca<sup>2+</sup>-free medium, whereas Oregon green 488 BAPTA-5N, AM (Molecular Probes; final concentration, 5 μM) was used for Ca<sup>2+</sup> imaging in normal medium. Cells were extensively washed after loading the Ca<sup>2+</sup> indicators in respective media, and then excited at 488 nm. The fluorescence images were acquired at the same rate using the same procedures as described in the previous paragraph.

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