Effects of cytochalasin treatment on short-term synaptic plasticity at developing neuromuscular junctions in frogs

Xin-hao Wang, James Q. Zheng and Mu-ming Poo*

Department of Biological Sciences, Columbia University, New York, NY 10027, USA

1. The role of actin microfilaments in synaptic transmission was tested by monitoring spontaneous and evoked transmitter release from developing neuromuscular synapses in *Xenopus* nerve–muscle cultures, using whole-cell recording of synaptic currents in the absence and presence of microfilament-disrupting agents cytochalasins B and D.

2. Treatment with cytochalasins resulted in disruption of microfilament networks in the growth cone and the presynaptic nerve terminal of spinal neurons in *Xenopus* nerve–muscle cultures, as revealed by rhodamine–phalloidin staining.

3. The same cytochalasin treatment did not significantly affect the spontaneous or evoked synaptic currents during low-frequency stimulation at 0·05 Hz in these *Xenopus* cultures. Synaptic depression induced by high-frequency (5 Hz) stimulation, however, was reduced by this treatment. Paired-pulse facilitation for short interpulse intervals was also increased by the treatment.

4. These results indicate that disruption of microfilaments alters short-term changes in transmitter release induced by repetitive activity, without affecting normal synaptic transmission at low frequency.

5. Our results support the notion that actin microfilaments impose a barrier for mobilization of synaptic vesicles from the reserve pool, but do not affect the exocytosis of immediately available synaptic vesicles at the active zone.

Neurotransmitter secretion from the presynaptic nerve terminal occurs with specific temporal and spatial characteristics that are determined by the organization of the secretory machinery in the presynaptic nerve terminal. Of particular importance are the distribution and the availability of synaptic vesicles for exocytosis at the site of secretion, or the active zone. Ultrastructural studies of the active zone showed the formation of a network of actin filaments in association with clusters of synaptic vesicles (Hirokawa, Sobue, Kanda, Harada & Yorifuji, 1989; Hirokawa, 1991). The synaptic vesicles may be attached to actin filaments through synapsin I, a synapse-specific phosphoprotein that binds to both actin filaments and synaptic vesicles (Benfenati, Valtorta, Chieregatti & Greengard, 1992; Greengard, Valtorta, Czernik & Benfenati, 1993). The actin-binding activity of synapsin I *in vitro* can be abolished through phosphorylation by Ca^{2+}–calmodulin-dependent protein kinase II (CaMKII). It has been proposed that a Ca^{2+}-dependent reversible linkage of synaptic vesicles to actin filaments via synapsins regulates the availability of synaptic vesicles for exocytosis (De Camili, Benfenati, Valtorta & Greengard, 1990; Valtorta, Benfenati & Greengard, 1992; Greengard *et al.* 1993). This hypothesis was supported by the findings that injection of dephosphosynapsin I, which promotes vesicle association with actin filaments, into presynaptic nerve terminals inhibited evoked transmitter release, while injection of CaMKII facilitated the release (Llinás, McGuinness, Leonard, Sugimori & Greengard, 1985; Lin, Sugimori, Llinás, McGuinness & Greengard, 1990; Hackett, Cochran, Greenfield, Brosius & Ueda, 1990; Llinás, Gruner, Sugimori, McGuinness & Greengard, 1991). Thus, transmitter secretion is likely to depend on the integrity of actin filaments in the presynaptic nerve terminal.

By staining with rhodamine-labelled phalloidin, a cycling of fluorescence intensity was observed when mouse brain synaptosomes were depolarized by high $[K^+]$ (Bernstein & Bamburg, 1989). DNAase I and phalloidin, drugs that affect actin assembly and disassembly, affect the K^+-evoked noradrenaline release from these synaptosomes (Bernstein

* To whom correspondence should be addressed at the Department of Biology, University of California, San Diego, La Jolla, CA 92093, USA.

& Bamburg, 1989). Regulated secretion in several other secretory cells has also been correlated with reorganization of cytoskeletal structures (Trifaró & Vitale, 1993). Furthermore, destabilization of actin filaments in permeabilized chromaffin cells by cytochalasin D or DNAase I potentiated Ca^{2+}-dependent secretion of catecholamine (Lelkes, Friedman, Rosenheck & Oplatka, 1986; Sontag, Aunis & Bader, 1988). Inhibition of actin polymerization in PC12 cells by botulinum C2 toxin also potentiated Ca^{2+}-dependent noradrenaline release (Matter, Dreyer & Aktories, 1989). These results have led to the proposal that the actin cytoskeleton acts as a barrier for exocytosis (Linstedt & Kelly, 1987; Aunis & Bader, 1988; Trifaró & Vitale, 1993; Vitale, Seward & Trifaró, 1995). Since some natural excitatory signals, e.g. membrane depolarization and nicotine, can affect the organization of actin filaments, it is possible that the actin cytoskeleton may also be actively involved in regulating transmitter release from the presynaptic nerve terminal.

Despite substantial biochemical and cytological evidence for possible roles of actin filaments in transmitter release, direct demonstration that actin filaments affect synaptic transmission at intact synapses under physiological conditions has not been reported. In the present study of developing *Xenopus* neuromuscular synapses, spontaneous and evoked ACh secretion triggered by low-frequency presynaptic stimulation were not affected by cytochalasins. However, we found that cytochalasins significantly affected evoked secretion during transmission at a higher frequency. The tetanus-induced synaptic depression was reduced and the paired-pulse facilitation was increased. These results support the notion that actin filaments do not affect the exocytosis of immediately releasable synaptic vesicles, but serve to restrain the vesicles in the reserve pool. Disruption of actin filaments mobilizes the vesicular supply, an effect that can be revealed only when the demand for evoked transmitter secretion is elevated.

METHODS

Culture and chemical preparations

Xenopus nerve–muscle cultures were prepared as previously reported (Spitzer & Lamborghini, 1976; Anderson, Cohen & Zorychta, 1977; Tabti & Poo, 1991). Briefly, the neural tube and the associated myotomal tissue of 1-day-old *Xenopus* embryos (stages 20–22; Nieuwkoop & Faber, 1967) were dissociated in Ca^{2+}- and Mg^{2+}-free saline supplemented with EDTA. The cells were plated on clean glass coverslips and used for experiments after 24 h at room temperature (20–22 °C). The culture medium consisted of 50% (v/v) Ringer solution (115 mM NaCl, 2 mM $CaCl_2$, 2·5 mM KCl, 10 mM Hepes, pH 7·6), 49% L-15 Leibovitz medium (Sigma), and 1% fetal bovine serum (Gibco). Cytochalasins B (CB) and D (CD) were obtained from Sigma, and dimethyl sulphoxide (DMSO) was from Aldrich Chemical Company, Inc. Cytochalasins were first dissolved in DMSO solution at 5 mg ml^{-1}, then diluted to 200 μg ml^{-1} for CB and 100 μg ml^{-1} for CD with Ringer solution before bath application to the culture.

Electrophysiology

Gigaohm-seal whole-cell recording and patch-clamp recording were performed as described previously (Hamill, Marty, Neher, Sakmann & Sigworth, 1981; Evers, Laser, Sun & Poo, 1989). Synaptic currents were recorded from innervated myocytes by whole-cell recording in voltage-clamp mode. The intrapipette solution contained 150 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$, and 10 mM Hepes buffer (pH 7·2). Evoked synaptic currents were elicited by stimulating presynaptic neurons at the soma with fire-polished glass microelectrodes (tip diameter, 2–3 μm). The pipette was filled with Ringer solution. For suprathreshold stimulation of the neuron, a square current pulse of 0·5 ms duration and 0·2–2 μA amplitude was applied through the pipette. Such currents consistently induce twitch contraction of the muscle cell when applied to the soma of the innervating neuron. When the myocyte was voltage clamped at the resting potential, however, no contraction was induced by neuronal stimulation. In all recordings, membrane currents were monitored by a patch-clamp amplifier (List EPC-7). The data were digitized (DR384; Neurodata, NY, USA) and stored on magnetic videotape for later playback onto a storage oscilloscope (Tektronix 5113) or an oscillographic recorder (Gould RS3200). All data were analysed using SCAN software (kindly provided by Dr J. Dempster, Strathclyde University, UK) for amplitude, frequency and time course of spontaneous synaptic currents (SSCs).

Fluorescence imaging of actin filaments

Xenopus nerve–muscle cultures were first fixed with 4% paraformaldehyde in cacodylic buffer (0·1 M cacodylic acid, 0·1 M sucrose, pH 7·2) for 20 min. The cells were then permeabilized in 0·1% Triton X-100 in Ringer solution for 5 min. After washing with Ringer solution, the cells were incubated with rhodamine-conjugated phalloidin (Molecular Probes, Inc., 1:100 dilution in Ringer solution from the stock) for 10 min. To facilitate visualization of the presynaptic nerve terminal without fluorescence interference from the postsynaptic (spherical) myocyte, the latter was removed by a micropipette after fixation, prior to staining. Removing the myocyte did not significantly affect the staining pattern of the nerve terminal, since we found that, in most cases, the myocytes were very easily removed after fixation without inducing visible movement of the nerve terminal. Cells were examined under an inverted fluorescence microscope (Axiovert; Zeiss) equipped with a cooled-CCD camera-based imaging system (Star I CCD camera; Photometrics, Inc., Tucson, AZ, USA). A ×40 0·9 NA water-immersion lens was used to visualize the actin staining in the neuron. Fluorescence images were acquired through the CCD camera and saved on disk in a digital format for contrast enhancement and printing. In some cases, fluorescence images were acquired through an intensified CCD video camera (Quantex, Inc., Grass Valley, CA, USA) and recorded on videotape before being digitized for storage and processing.

All values are given as means ± S.E.M., levels of significance were determined using Student's paired t test.

RESULTS

Effects of cytochalasins on actin filaments

The distribution of actin filaments in the cytoplasm of cultured *Xenopus* spinal neurons was studied by staining with rhodamine–phalloidin (Wulf, Deboben, Bautz,

Table 1. Effects of cytochalasin B on spontaneous synaptic currents

	Mean frequency (events min^{-1})	Mean amplitude (pA)	Rise time (ms)	Half-decay time (ms)	No. synapses examined
Before CB	10 ± 4	572 ± 92	1·6 ± 0·3	3·6 ± 0·3	7
After CB	15 ± 9	493 ± 87	1·6 ± 0·3	3·7 ± 0·5	7
Before DMSO	11 ± 6	536 ± 67	2·1 ± 0·3	3·5 ± 0·3	6
After DMSO	12 ± 7	550 ± 101	2·4 ± 0·3	4·2 ± 0·4	6

The mean value of each parameter was determined over a 10–20 min period for each synapse before averaging.

Faulstich & Wieland, 1979; Faulstich, Trischmann & Mayer, 1983) after fixation and permeabilization of the cells. Both isolated spinal neurons and neurons that had innervated co-cultured myocytes were examined. In the absence of cytochalasin treatment, relatively uniform staining of actin filaments was observed along the neuritic processes, at the growth cones and neurite terminals (Fig. 1*A* and *C*). Treatment of the culture with 20 μM CB for 10 min prior to the staining resulted in disruption of the filamentous structure, leading to punctuate staining in all regions of the neuron (Fig. 1*B* and *D*). Similar disruption of actin filament staining was observed after 10 min incubation with 10 μM CD (data not shown).

Effects on spontaneous ACh secretion

SSCs were recorded from innervated myocytes in 1-day-old *Xenopus* nerve–muscle cultures using the gigaohm-seal whole-cell recording method (Hamill *et al.* 1981). After 10 min of recording (control), cytochalasins were bath applied and recording continued for up to 40 min. At a bath CB concentration of 20 μM, which is known to disrupt actin filaments in these *Xenopus* neurons and to inhibit the association and dissociation events at the barbed end of actin filaments *in vitro* (Bonder & Mooseker, 1986), we found no significant change in frequency, mean amplitude, rise time and decay time of SSCs after CB treatment (see Table 1). A typical recording is shown in Fig. 2*A*. Figure 2*B*

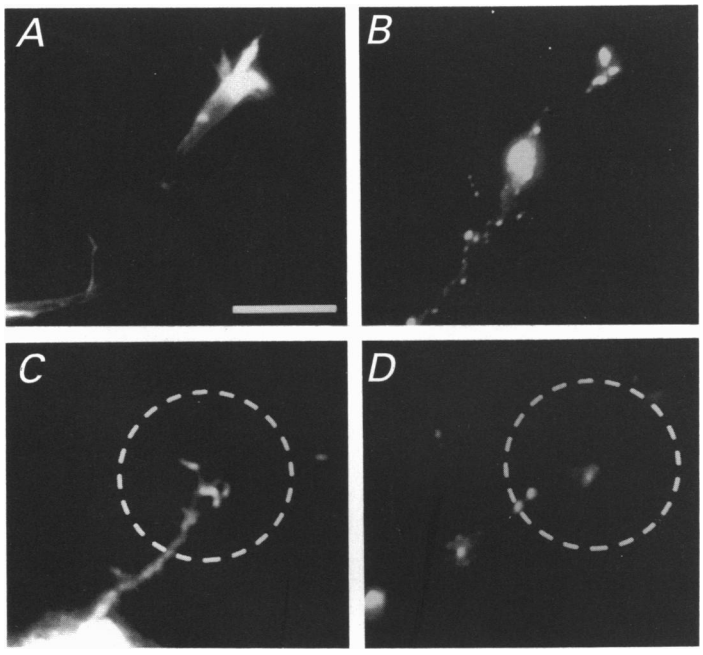

Figure 1. Effect of CB treatment on the organization of actin filaments

Xenopus cultures were stained for actin filaments with rhodamine–phalloidin. Examples of fluorescence images of neurites with a free growth cone (*A* and *B*) and neurites that had innervated a spherical myocyte (*C* and *D*) in 1-day-old *Xenopus* nerve–muscle cultures. *A* and *C*, control cultures not treated with CB. *B* and *D*, cultures treated with CB (20 μM) for 10 min. Dashed circles mark the positions of the postsynaptic myocytes, which were removed by a micropipette prior to staining. Scale bar, 20 μm.

depicts changes in mean SSC frequency at various times before and after application of CB. In control experiments DMSO (0·2% final bath concentration) was added without CB. Analysis of the amplitude distribution of SSCs (Fig. 2*C*) also showed that no significant change was induced by CB. Application of CD (10 μM), which is known to be more potent in disrupting actin filaments (Cooper, 1987), was similarly ineffective in changing the frequency or amplitude of SSCs (data not shown).

Effects on evoked ACh release at low-frequency transmission

Presynaptic neurons were stimulated extracellularly at the soma to fire action potentials at a low frequency (0·05 Hz), and evoked synaptic currents (ESCs) were recorded from the postsynaptic myocyte before and after bath application of cytochalasins. An example of the recording is shown in Fig. 3*A*. In the absence of the cytochalasin, the mean amplitude of ESCs exhibited a gradual reduction after repetitive test stimuli, a phenomenon of synaptic depression known to occur at developing synapses (Lo & Poo, 1991; Lohof, Ip & Poo, 1993). In cultures treated with CB (20 μM), no significant effect was found when the mean normalized ESC amplitudes were compared with those observed in control cultures treated with DMSO (see Fig. 3*B*). However, in a minority of cases, the amplitude of ESCs after CB treatment was found to increase within a few minutes. To illustrate the variability of results, a scatter plot was made to show the ratio of mean ESC amplitude 5–10 min following CB application to that before (Fig. 3*C*). Despite apparent increase in ESC amplitudes in a few cases, the mean was not significantly different from that of the controls ($P > 0·05$). Table 2 summarizes the effects of CB on the mean amplitude, rise time, and half-decay time of ESCs, as well as on the delay of onset of postsynaptic response following the stimulus. Similar treatment with CD (10 μM) was also without effect (data not shown). We thus concluded that the overall effect of cytochalasins on the evoked ACh secretion was not significant at low-frequency synaptic transmission.

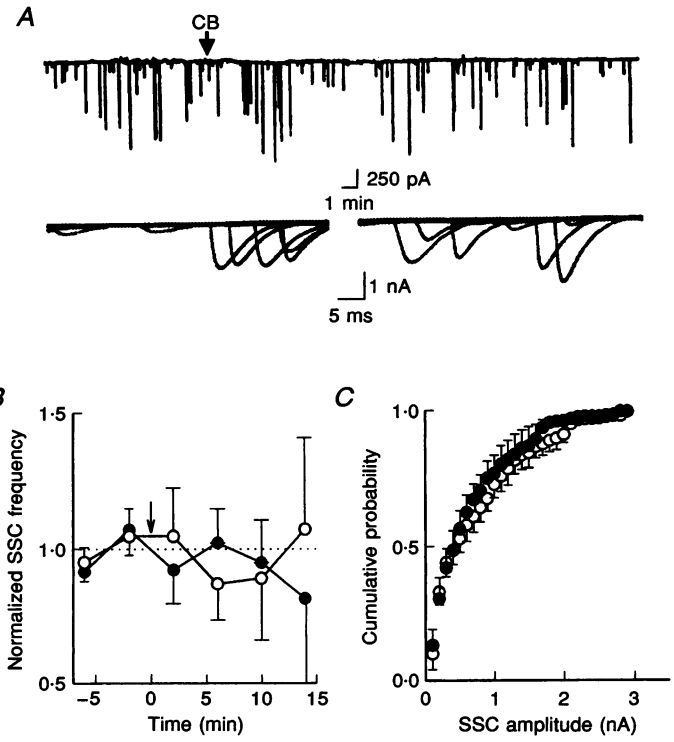

Figure 2. Absence of CB effect on spontaneous ACh release in 1-day-old *Xenopus* cultures

A, the continuous trace depicts the membrane current recorded from an innervated myocyte before and after bath application of CB (arrow indicates onset; 20 μM final concentration). Downward deflections represent SSCs (command potential (V_c), −70 mV; filtered at 150 Hz). Lower traces are samples of SSCs shown at a higher time resolution (filtered at 2·5 kHz). *B*, changes in mean SSC frequency before and after CB treatment. SSC frequency is normalized to the frequency before drug treatment. ●, experiments in which CB was applied (20 μM final concentration, $n = 8$); ○, experiments in which DMSO alone was applied ($n = 7$). Arrow marks the onset of drug application. *C*, distribution of SSC amplitude before (○) and after (●) CB treatment. The cumulative probability refers to the proportion of total events with amplitude smaller than a given amplitude. SSC data from *B* were used for the amplitude analysis. Error bars represent S.E.M.

Table 2. Effects of cytochalasin B on evoked synaptic currents

	Mean amplitude (nA)	Rise time (ms)	Half-decay time (ms)	Synaptic delay (ms)	No. synapses examined
Before CB	4·2 ± 0·6	1·1 ± 0·1	5·6 ± 0·9	1·4 ± 0·3	6
After CB	3·0 ± 0·5	1·0 ± 0·1	5·0 ± 1·1	1·4 ± 0·2	6
Before DMSO	3·3 ± 0·5	1·3 ± 0·2	5·3 ± 0·8	1·8 ± 0·2	5
After DMSO	2·4 ± 0·4	1·4 ± 0·2	6·6 ± 1·3	1·9 ± 0·2	5

Synaptic delay refers to the time between the onset of stimulus (0·5 ms duration) and the onset of the ESC. The mean value of each parameter was determined over a 10–20 min period for each synapse before averaging.

Effects on synaptic depression during tetanic stimulation

Depression of transmitter secretion during prolonged tetanic stimulation at high frequency is a common short-term plasticity of many synapses, reflecting the depletion of the vesicular supply of transmitters (for review see Zucker, 1989). For neuromuscular synapses in 1-day-old *Xenopus* cultures, tetanic stimulation at 5 Hz induced a 23 ± 4% ($n = 65$) reduction in mean ESC amplitude after 30 s. This tetanus-induced depression was found to be

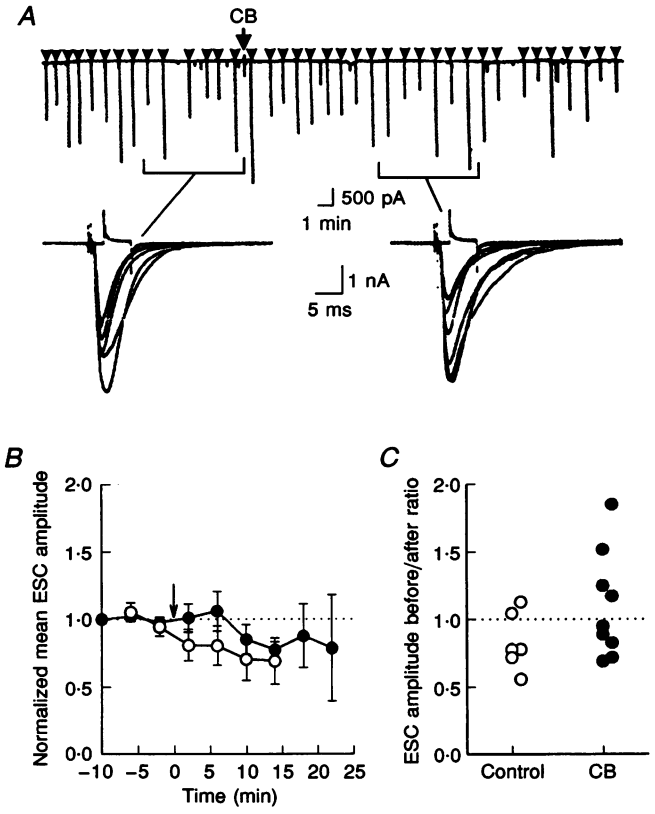

Figure 3. Absence of the CB effect on evoked ACh release during low-frequency stimulation

A, the continuous trace depicts membrane current recorded from an innervated myocyte (V_c, −70 mV; filtered at 150 Hz) before and after CB treatment (arrow indicates onset). ESCs were elicited at low frequency at the times indicated by arrowheads. Lower traces are the samples of ESCs shown at a higher time resolution (filtered at 2·5 kHz) for the recording periods indicated. *B*, the mean ESC amplitude was normalized to the value before drug treatment. ●, experiments in which CB was applied (20 μM final concentration, $n = 9$). ○, control experiments in which DMSO alone was applied ($n = 6$). *C*, scatter plot of the ratio of ESC amplitudes for all individual cases (same data as for *B*). The ratio is determined by dividing the mean ESC amplitude between 5–10 min after the drug treatment with that before the treatment.

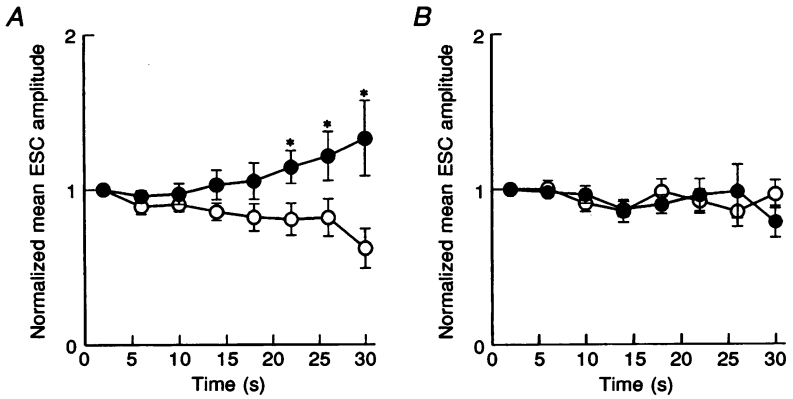

Figure 4. The effect of CB on synaptic depression during tetanic stimulation

A, changes in ESC amplitude during 5 Hz tetanic stimulation. ○, mean ESC amplitude during tetanic stimulation before CB treatment ($n = 6$). ●, ESC amplitude change for the same set of synapses after CB treatment (20 μM final concentration, $n = 6$). Mean ESC amplitudes over 4 s intervals are normalized to the mean ESC amplitude over the first 4 s after the onset of tetanus. The normalized amplitudes at 22, 26 and 30 s (asterisks) show significant differences before and after CB treatment ($P < 0{\cdot}05$). *B*, results from control experiments in which DMSO was applied without CB. ○, before DMSO; ●, after DMSO.

reduced after CB treatment for 10 min. The mean ESC amplitude, normalized to the mean amplitude of the initial twenty ESCs, was determined for the first 30 s during tetanus (Fig. 4). It is apparent that, at this frequency of stimulation, synaptic depression was replaced by a slight potentiation after CB treatment. Significant difference between normalized mean ESC amplitudes before and after the CB treatment occurred after 20 s (Fig. 4*A*, $P < 0{\cdot}05$). In parallel control experiments, we applied the same amount of solvent (DMSO) as that used in the CB treatment and found no change in the tetanus-induced depression before and after application (Fig. 4*B*).

To estimate the effect of applying different concentrations of cytochalasins on synaptic depression, we determined the changes in synaptic depression by subtracting the normalized mean ESC amplitude after 30 s of tetanus before the drug treatment from that observed after the treatment at each synapse. As shown in Fig. 5, significant CB effect was detected at a drug concentration above 15 μM ($P < 0{\cdot}05$), while significant CD effect appeared at 10 μM.

Effects on paired-pulse facilitation

Paired-pulse facilitation (PPF), the increase in transmitter release evoked by the second stimulus during a twin-pulse stimulation, reflects a form of short-term plasticity at many synapses (Katz & Miledi, 1968; Mallart & Martin, 1968; Zucker, 1989). To examine the effect of cytochalasins on PPF, twin pulses with different interpulse intervals, ranging from 20 to 200 ms, were applied to the soma of presynaptic neurons, and the ratio of ESC amplitudes between the second and first ESCs (the paired-pulse ratio, PPR) was determined. Results are shown in Fig. 6. There was substantial variation in PPR at *Xenopus* neuromuscular synapses, but no statistically significant PPF was observed in any interpulse interval. However, after CB

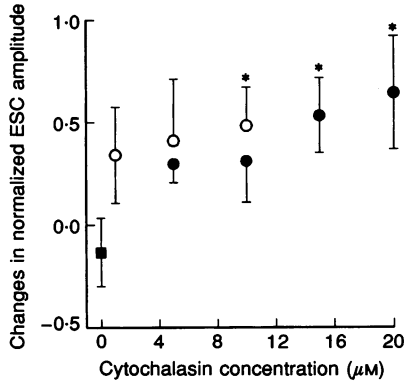

Figure 5. Dose dependence of the cytochalasin effect during tetanic stimulation

Each data point refers to the mean difference between the normalized mean ESC amplitudes at the end of 30 s tetanus before and after drug treatment. ●, CB treated; ○, CD treated; ■, DMSO alone. Significant effects compared with the control were found for CB at 15 and 20 μM, and for CD at 10 μM (* $P < 0{\cdot}05$).

(20 μm) treatment for 10 min, the synapses displayed small but significant PPF for interpulse intervals at 20 and 30 ms with PPRs of 1·11 ± 0·04 and 1·26 ± 0·05 ($n = 14$), respectively. These values were significantly different from those observed in the same group of synapses (0·99 ± 0·04 and 1·03 ± 0·08) before the drug treatment ($P < 0·05$). No significant difference was found in any other interval tested. Similar results were also obtained with CD treatment (10 μM).

DISCUSSION

Previous evidence suggests that there are two pools of synaptic vesicles within the nerve terminal: vesicles docked at the presynaptic membrane which are immediately available for release, and vesicles that are recruited to the release site for vesicle replenishment during continued transmitter secretion (for reviews see Hirokawa, 1991; Greengard *et al.* 1993). The number of vesicles released per stimulus depends, in part, on the size of the immediately releasable pool, which is in turn determined by the number of docking sites at the plasma membrane and the rate of vesicle mobilization from the reserve pool. It has been shown that during repetitive stimulation the amount of transmitter released per stimulus is proportional to the size of the reserve pool (Koenig, Kosaka & Ikeda, 1989; Hurlbut, Iezzi, Fesce & Ceccarelli, 1990). These findings suggest that, when the demand for transmitter secretion increases, vesicle mobilization may become rate limiting in the secretion process. In the present study, we have shown that cytochalasins had no significant effect on spontaneous and evoked ACh release at low-frequency transmission, but significantly enhanced the evoked secretion during tetanic or paired-pulse stimulation at short interpulse intervals. These results are consistent with the notion that cytochalasins do not affect the secretion of the docked vesicles, but can mobilize vesicles from the reserve pool. During spontaneous and low-frequency evoked release, changes in the rate of vesicle mobilization will not affect transmitter release if most available docking sites are occupied and the normal rate of mobilization is rapid enough to replenish the vacant docking sites. Under high-frequency stimulation, the rate of vesicle mobilization becomes important because of the demand for more rapid

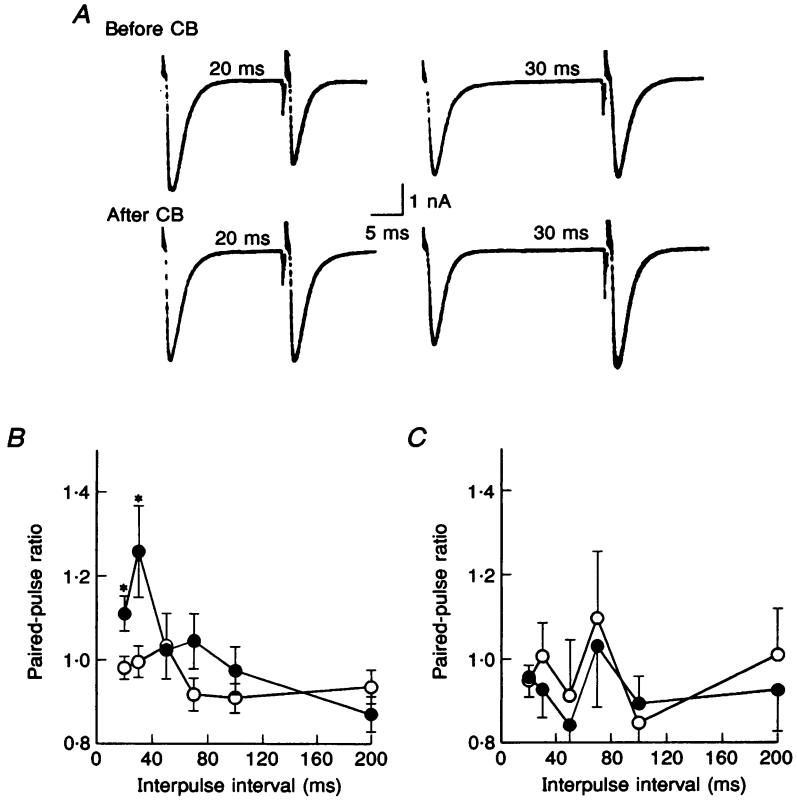

Figure 6. The CB effect on paired-pulse facilitation

A, samples of ESCs induced by paired pulses before and after CB treatment (20 μM) are shown. *B*, changes in the PPR after CB treatment. The PPR was determined by the amplitude of the second ESC divided by that of the first ESC. Mean PPRs ($n = 14$) with different interpulse intervals before (○) and after (●) CB treatment are shown. Points with significant difference between the values before and after the CB treatment are marked with asterisks ($P < 0·05$, paired t test). *C*, control experiments with treatment of DMSO alone ($n = 5$). ○, before DMSO; ●, after DMSO.

vesicle replenishment. In untreated synapses, the absence of PPF at short interpulse intervals suggests that the effect of elevated Ca^{2+} which resulted from the first impulse may be masked by a substantial depletion of the docked vesicles after the first evoked release. The increased rate of mobilization after the cytochalasin treatment facilitated more rapid replenishment within the interpulse interval, thus allowing the appearance of facilitated secretion during the second pulse, and this effect would be more pronounced at short interpulse intervals.

Actin assembly and disassembly is a dynamic process in the cell. Cytochalasins can bind to and inhibit the association and dissociation events at the barbed end of the actin filament (Bonder & Mooseker, 1986; Cooper, 1987), resulting in shortening of the filament and disruption of the filamentous network. Such disruption could mobilize synaptic vesicles restrained within the network. This is consistent with the model in which synaptic vesicles are anchored to the actin filament through synapsins, so that phosphorylation of synapsins frees the vesicles from the anchor, allowing vesicle mobilization for immediate release (De Camili *et al.* 1990; Valtorta *et al.* 1992; Greengard *et al.* 1993). Thus, in addition to the regulation by synapsin phosphorylation, transmitter secretion may also be regulated by dynamic changes of the actin filament network. The latter possibility is supported by the findings that, under high $[K^+]$ stimulation of mouse brain synaptosomes, actin depolymerization is necessary for normal noradrenaline release, and repolymerization limits the transmitter release (Berstein & Bamburg, 1989). If actin filaments play an active role in regulating transmitter secretion, rather than serving as a passive barrier for vesicle mobilization, many other regulatory proteins involved in the assembly and disassembly of actin filaments, such as profilin, gelsolin, and scinderin (Yin, Albrecht & Fattoum, 1981; Nishida, Maekawa & Sakai, 1984; Rodríguez Del Castillo *et al.* 1990) may all play important regulatory roles in transmitter secretion.

Betz & Henkel (1994) used a fluorescence dye FM1-43 to monitor the trafficking of synaptic vesicles at frog cutaneous pectoris neuromuscular synapses. Based on the intensity and distribution of fluorescent spots, they concluded that cytochalasin D treatment had no significant effect on the mobilization or movement of the synaptic vesicle. This appears to be inconsistent with our present findings. While it is possible that the organization of actin filaments at developing neuromuscular synapses examined in the present study is more susceptible to disruption by cytochalasins, the electrophysiological assay used here may be more sensitive to changes in vesicle mobilization. Different synapses may also differ in their normal rate of vesicle mobilization and in the conditions under which cytochalasin effects can be revealed.

Anderson, M. J., Cohen, M. W. & Zorychta, E. (1977). Effects of innervation on the distribution of acetylcholine receptors on cultured muscle cells. *Journal of Physiology* **268**, 731–756.

Aunis, D. & Bader, M. F. (1988). The cytoskeleton as a barrier to exocytosis in secretory cells. *Journal of Experimental Biology* **139**, 252–266.

Benfenati, F., Valtorta, F., Chieregatti, E. & Greengard, P. (1992). Interaction of free and synaptic vesicle-bound synapsin I with F-actin. *Neuron* **8**, 377–386.

Bernstein, B. W. & Bamburg, J. R. (1989). Cycling of actin assembly in synaptosomes and neurotransmitter release. *Neuron* **3**, 257–265.

Betz, W. J. & Henkel, A. W. (1994). Okadaic acid disrupts clusters of synaptic vesicles in frog motor nerve terminals. *Journal of Cell Biology* **124**, 843–854.

Bonder, E. M. & Mooseker, M. S. (1986). Cytochalasin B slows but does not prevent monomer addition at the barbed end of the actin filament. *Journal of Cell Biology* **102**, 282–288.

Cooper, J. A. (1987). Effects of cytochalasin and phalloidin on actin. *Journal of Cell Biology* **105**, 1473–1478.

De Camili, P., Benfenati, F., Valtorta, F. & Greengard, P. (1990). The synapsins. *Annual Review of Cell Biology* **6**, 433–460.

Evers, J., Laser, M., Sun, Y., Xie, Z. & Poo, M.-m. (1989). Studies of nerve–muscle interactions in *Xenopus* cell culture: analysis of early synaptic currents. *Journal of Neuroscience* **9**, 1523–1539.

Faulstich, H., Trischmann, H. & Mayer, D. (1983). Preparation of tetramethylrhodaminyl–phalloidin and uptake of the toxin into short-term cultured hepatocytes by endocytosis. *Experimental Cell Research* **144**, 73–82.

Greengard, P., Valtorta, F., Czernik, A. J. & Benfenati, F. (1993). Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science* **259**, 780–785.

Hackett, J. T., Cochran, S. L., Greenfield, L. J., Brosius, D. C. & Ueda, T. (1990). Synapsin I injected presynaptically into goldfish Mauthner axons reduces quantal synaptic transmission. *Journal of Neurophysiology* **63**, 701–706.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

Hirokawa, N. (1991). Molecular architecture and dynamics of the neuronal cytoskeleton. In *The Neuronal Cytoskeleton*, ed. Burgoyne, R. A., pp. 288–321. Wiley-Liss, New York.

Hirokawa, N., Sobue, K., Kanda, K., Harada, A. & Yorifuji, H. (1989). The cytoskeletal architecture of the presynaptic terminal and molecular structure of synapsin I. *Journal of Cell Biology* **108**, 111–126.

Hurlbut, W. P., Iezzi, N., Fesce, R. & Ceccarelli, B. (1990). Correlation between quantal secretion and vesicle loss at the frog neuromuscular junction. *Journal of Physiology* **425**, 501–526.

Katz, B. & Miledi, R. (1968). The role of calcium in neuromuscular facilitation. *Journal of Physiology* **195**, 481–492.

Kelly, R. B. (1993). Storage and release of neurotransmitters. *Cell* **72** (*Cell* **72**/*Neuron* **10** review supplement), 43–53.

Koenig, J. H., Kosaka, T. & Ikeda, K. (1989). The relationship between the number of synaptic vesicles and the amount of transmitter released. *Journal of Neuroscience* **9**, 1937–1942.

LELKES, P. I., FRIEDMAN, J. E., ROSENHECK, K. & OPLATKA, A. (1986). Destabilization of actin filaments as a requirement for the secretion of catecholamines from permeabilized chromaffin cells. *FEBS Letters* **208**, 357–363.

LIN, J. W., SUGIMORI, M., LLINÁS, R., MCGUINNESS, T. L. & GREENGARD, P. (1990). Effects of synapsin I and calcium/calmodulin-dependent protein kinase II on spontaneous neurotransmitter release in the squid giant synapse. *Proceedings of the National Academy of Sciences of the USA* **87**, 8257–8261.

LINSTEDT, A. D. & KELLY, R. B. (1987). Overcoming barriers to exocytosis. *Trends in Neurosciences* **10**, 446–448.

LLINÁS, R., GRUNER, J. A., SUGIMORI, M., MCGUINNESS, T. L. & GREENGARD, P. (1991). Regulation by synapsin I and Ca^{2+}–calmodulin-dependent protein kinase II of transmitter release in squid giant synapse. *Journal of Physiology* **436**, 257–282.

LLINÁS, R., MCGUINNESS, T. L., LEONARD, C. S., SUGIMORI, M. & GREENGARD, P. (1985). Intraterminal injection of synapsin I or calcium/calmodulin-dependent protein kinase II alters neurotransmitter release at the squid giant synapse. *Proceedings of the National Academy of Sciences of the USA* **82**, 3035–3039.

LO, Y. J. & POO, M.-M. (1991). Activity-dependent synaptic competition *in vitro*: heterosynaptic suppression of developing synapses. *Science* **254**, 1019–1022.

LOHOF, A. M., IP, N. Y. & POO, M.-M. (1993). Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF. *Nature* **363**, 350–353.

MALLART, A. & MARTIN, A. R. (1968). The relation between quantum content and facilitation at the neuromuscular junction of the frog. *Journal of Physiology* **196**, 593–604.

MATTER, K., DREYER, F. & AKTORIES, K. (1989). Actin involvement in exocytosis from PC12 cells: studies of the influence of Botulinum C2 toxin on stimulated noradrenaline release. *Journal of Neurochemistry* **52**, 370–376.

NIEUWKOOP, P. D. & FABER, J. (1967). Normal table of *Xenopus laevis*, 2nd edn. North-Holland Publishing Company, Amsterdam.

NISHIDA, E., MAEKAWA, S. & SAKAI, H. (1984). Characterization of the action of porcine brain profilin on actin polymerization. *Journal of Biochemistry* **95**, 399–404.

RODRÍGUEZ DEL CASTILLO, A., LEMAIRE, S., TCHAKAROV, L., JEYAPRAGASAN, M., DOUCET, J. P., VITALE, M. L. & TRIFARÓ, J. M. (1990). Chromaffin cell scinderin, a novel calcium-dependent actin filament-severing protein. *EMBO Journal* **9**, 43–52.

SONTAG, J. M., AUNIS, D. & BADER, M. F. (1988). Peripheral actin filaments control calcium-mediated catecholamine release from streptolysin-O-permeabilized chromaffin cells. *European Journal of Cell Biology* **46**, 316–326.

SPITZER, N. C. & LAMBORGHINI, J. C. (1976). The development of the action potential mechanism of amphibian neurons isolated in culture. *Proceedings of the National Academy of Sciences of the USA* **73**, 1641–1645.

TABTI, N. & POO, M.-M. (1991). Culturing spinal cord neurons and muscle cells from *Xenopus* embryos. In *Culturing Nerve Cells*, ed. BANKER, G. & GOSLIN, K., pp. 137–154. MIT Press, Cambridge, MA, USA.

TRIFARÓ, J.-M. & VITALE, M. L. (1993). Cytoskeleton dynamics during neurotransmitter release. *Trends in Neurosciences* **16**, 466–472.

VALTORTA, F., BENFENATI, F. & GREENGARD, P. (1992). Structure and function of the synapsins. *Journal of Biological Chemistry* **267**, 7195–7198.

VITALE, M. L., SEWARD, E. P. & TRIFARÓ, J.-M. (1995). Chromaffin cell cortical actin network dynamics control the size of the release-ready vesicle pool and the initial rate of exocytosis. *Neuron* **14**, 353–363.

WULF, E., DEBOBEN, A., BAUTZ, F. A., FAULSTICH, H. & WIELAND, T. (1979). Fluorescent phallotoxin, a tool for the visualization of cellular actin. *Proceedings of the National Academy of Sciences of the USA* **76**, 4498–4502.

YIN, H. L., ALBRECHT, J. & FATTOUM, A. (1981). Identification of gelsolin, a Ca^{2+}-dependent regulatory protein of actin gelsol transformation, its intercellular distribution in a variety of cells and tissues. *Journal of Cell Biology* **91**, 901–906.

ZUCKER, R. S. (1989). Short-term synaptic plasticity. *Annual Review of Neuroscience* **12**, 13–31.

Acknowledgements

This work was supported by grants from the National Science Foundation (IBN 22106) and Office of Naval Research (N00014-90-J1865).

Received 14 March 1995; accepted 2 August 1995.