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# Inhibition of quantal release from motor nerve by wortmannin

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**1** The effects of wortmannin, an inhibitor of phosphatidylinositol (PI) kinases and myosin light chain kinase, on the quantal release of neurotransmitter from mouse phrenic nerve were investigated.

**2** Wortmannin (10–100 $\mu$M) initially enhanced, thereafter progressively depressed spontaneous quantal discharge (miniature endplate potential, mepp). The mean amplitude and the amplitude distribution of mepp were not altered.

**3** The compound inhibited and prevented the intensive quantal release evoked by high KC1 solution as well as the mepp burst induced by $\alpha$-latrotoxin, a polypeptide toxin that possesses $Ca^{2+}$-independent synaptic action to trigger quantal release. The inhibitory actions of wortmannin were partially reversible.

**4** Wortmannin depressed the amplitude of endplate potentials (epps) and increased the coefficient of variance of epps. The profile of epps in response to high frequency nerve stimulation exhibited fluctuations between run-down and run-up. The phenomenon is thus different from the consistency of run-up characteristic as the motor nerve $Ca^{2+}$ channel is blocked by $\omega$-agatoxin IVA.

**5** LY294002, another inhibitor of PI 3-kinase, raised mepp frequency without causing late phase suppressions. The compound did not inhibit KC1-, $\alpha$-latrotoxin- or nerve stimulation-evoked quantal release.

**6** The results suggest that wortmannin could depress quantal release beyond the step of $Ca^{2+}$ channel blockade, probably by interfering with the exocytotic cascade.

**Keywords:** $\alpha$-Latrotoxin; wortmannin; exocytosis; neuromuscular junction; miniature endplate potential

**Abbreviations:** epp, endplate potential; mepp, miniature endplate potential; PI, phosphatidylinositol

## Introduction

Under physiological conditions, the orthograde communication from nerve to skeletal muscle relies on sophisticated synaptic transmission. On the presynaptic side, the motor nerve terminal is endowed with secretory organelles and efficient machineries for packaging and release of the neurotransmitter–acetylcholine. The apposed postsynaptic muscle membrane is furnished with high density of nicotinic acetylcholine receptors for the generation of endplate potentials (epps). According to the hypothesis of quantal release, neurotransmission is achieved by the fusion of synaptic vesicles and nerve membrane with subsequent discharge of vesicular content–an exocytotic process involving complicated protein interactions and phospholipid rearrangement (Robinson & Martin, 1998; Fernández-Chacón & Südhof, 1999). The exocytosis can be facilitated by an appropriate elevation of intraterminal $Ca^{2+}$ concentration or by $\alpha$-latrotoxin, a protein component of black widow spider venom (Fesce *et al*., 1986; Rosenthal & Meldolesi, 1989). To maintain homeostasis and to replenish synaptic vesicles, the vesicular membrane intercalated with nerve membrane is retrieved by endocytosis (Heuser & Reese, 1973; Artalejo *et al*., 1995). Recent studies have revealed that specific proteins and phospholipids play important roles in the transport of membrane components and exocytotic release of bioactive molecules (De Camilli *et al*., 1996; Corvera & Czech, 1998). For example, myosin light chain kinase might mediate the exocytosis of catecholamine in chromaffin cell (Kumakura *et al*., 1994) and gonadotropin in pituitary cell (Rao *et al*., 1997), and phosphatidylinositol (PI) kinase might be implicated in insulin release (Straub & Sharp, 1996) and mast cell degranulation (Marquardt *et al*., 1996). We had characterized that wortmannin, an inhibitor of myosin light chain kinase and PI kinases (Nakanishi *et al*., 1992; Powis *et al*., 1994), interferes with the excitation-contraction coupling of skeletal muscle and depresses the contractile response (Hong & Chang, 1998). Here, we explore the presynaptic action of wortmannin in the mouse neuromuscular junction. The agent inhibits exocytosis evoked by nerve stimulation as well as $Ca^{2+}$-independent quantal release provoked by $\alpha$-latrotoxin.

## Methods

### *Motor nerve-skeletal muscle preparation*

Phrenic nerve-hemidiaphragms were isolated from mice (ICR strain, 20–25 g) killed by a heavy blow and exsanguination. Preparation was bathed in Tyrode solution (composition in mM: NaC1 137, KC1 2.8, $CaC1_2$ 1.8, $MgC1_2$ 1.1, $NaHCO_3$, 11.9, $NaH_2PO_4$ 0.33 and dextrose 11.2) maintained at 35–37°C with the pH adjusted at 7.3–7.4 by aeration with 5% $CO_2$ in $O_2$. Phrenic nerve was stimulated with supramaximal rectangular pulses of 0.03 ms duration. When appropriate, the organ bath solution was renewed with Tyrode solution every 15 min to washout wortmannin, $\alpha$-latrotoxin or high KC1.

### *Electrophysiological recording*

Glass microelectrodes for intracellular recording of miniature endplate potentials (mepps) and epps were filled with 3 M KCl

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(resistance 5–10 MΩ). To record epps, preparations were immobilized with $\mu$-conotoxin (Hong & Chang, 1995). Compound nerve action potentials were recorded extracellularly with a glass microelectrode (filled with 1 M NaCl, resistance 20–40 MΩ) pierced through the encapsulating sheath of the trunk of phrenic nerve. Membrane potentials and epps were d.c.-coupled whereas high gain signals of mepps and compound nerve action potentials were recorded in a.c. mode. Waveforms were digitized and displayed on a thermal recorder (Gould TA5000). The frequency response of the recording system was 10 kHz. Rise times (time-to-peak) and durations (the time spanning 20% peak amplitude) of mepps and epps were calculated. Epp amplitudes were corrected for non-linear summation, assuming 0 mV for the reversal potential, and then normalized to a resting membrane potential of −80 mV (c.f. Chang *et al.*, 1986).

### *Chemicals*

$\mu$-Conotoxin GIIIB was purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland), $\omega$-agatoxin IVA from Peptide Institute Inc. (Osaka, Japan) and $\alpha$-latrotoxin from Alomone Labs (Jerusalem, Israel). Wortmannin and 2-[4-morpholinyl]-8-phenyl-1[4H]-benzopyran-4-one (LY294002), from Sigma Chemical Company (St. Louis, U.S.A.), were dissolved in dimethylsulfoxide. The final concentration of the solvent in the organ bath was kept $<0.1\%$ (v $v^{-1}$) and the vehicle did not alter evoked and spontaneous quantal releases.

### *Statistics*

Mepp burst was defined as a gust of mepp-like discharges exceeding the basal level by either >30 events/s and persisting for at least 3 s, or >100 events/s lasting for longer than 0.3 s, followed by remission of the intensive firing. Large mepps (>4 mV or >2.5-fold average amplitude) with long rise-times (>2 ms) were included in the counting of discharge events but not in the estimation of mean amplitude. For each preparation, experiments were segmented into several parts (control, pretreatment, test or washout). For every portion, 4–7 different endplates were monitored. Each intracellular impalement was maintained for 5 min to sample mepps. Epp amplitude was the mean of 20 epps evoked at 0.3 Hz during the last minute of recording. The profile of epps in response to high frequency nerve stimulation (50–100 Hz for 2–4 s) was arbitrarily classified into three categories according to the ratio of steady state epps (the mean amplitude of the 16th to the 100th epps) relative to the first epp: run-down (when the value was <80%), run-up (when the value was >150%), or flat (between 80–150%). Data, expressed in means ± s.e., were pooled from 3–6 preparations. Differences between means were considered significant by Student's *t*-test with a *P* value less than 0.05.

## Results

### *Biphasic effects of wortmannin on mepps*

Firstly, the effects of wortmannin on spontaneous quantal discharge were investigated. In the *in vitro* condition, phrenic nerve discharged quantal events at 0.5–2 events/s for the initial 2 h and gradually to 2–3 events/s at 3–4 h (Figure 1). Addition of wortmannin (10–100 $\mu$M) elevated the mepp frequency up to 10 events/s (Figure 1) but mepp burst was never detected. The facilitation phase lasted for 1–2 h, thereafter mepp frequency decreased to 0.05–2 events/s at 3–4 h. The value is significantly less than the corresponding control. The onset and magnitude of facilitation and inhibition were related to the concentration of wortmannin. The suppression of mepp frequency was partially reversible, e.g. mepp frequency was <0.05 events/s 4 h after treatment with wortmannin 100 $\mu$M, however, after washout of wortmannin the frequency increased to 1–2 events/s. During the facilitation phase, the amplitude distribution of mepps was unchanged (Figure 1B). Even after long-term (3–6 h) treatment, wortmannin did not cause changes in mepp amplitude (1.5 ± 0.3 vs 1.7 ± 0.2 mV, $n=4$, 21–25 endplates) nor in mepp duration (3.9 ± 0.4 vs 3.4 ± 0.3 ms). In $Ca^{2+}$ -free Tyrode solution ($CaC1_2$ substituted by equi-moles of $MgC1_2$ or $CoCl_2$), wortmannin also produced biphasic changes in mepp frequency, though the facilitatory effect was less marked than that in normal Tyrode solution (not shown).

### *Depression of KCl-evoked quantal discharge*

In the second set of experiments, the effects of wortmannin on $Ca^{2+}$-dependent quantal release were studied. To compromise high KC1 solution-induced reduction of the electrochemical gradient for nicotinic receptor-cation channel, KC1 concentration was raised to 17 mM. Within 1 min of solution change to high KC1 medium, the muscle membrane depolarized by ~20 mV (−63.5 ± 2.9 vs −82.3 ± 2.1 mV, $n=5$, 26 endplates). At the same time mepp amplitude decreased moderately (1.0 ± 0.2

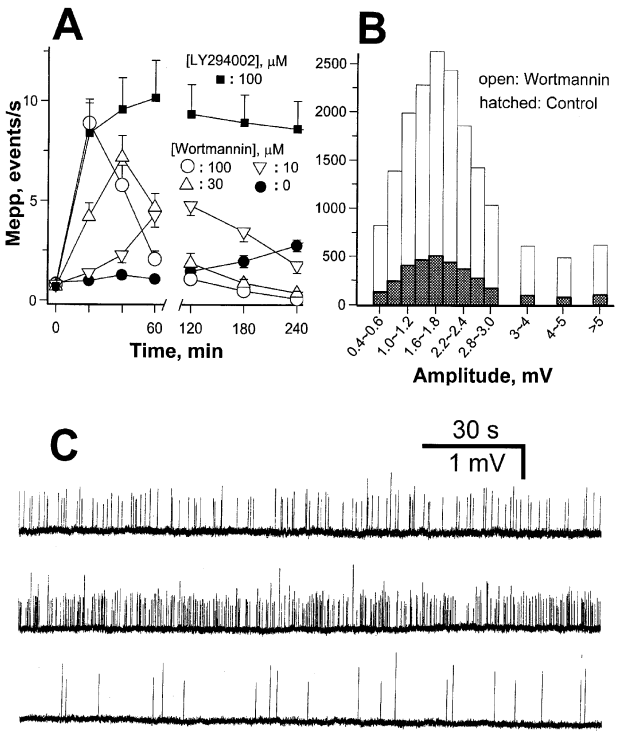


**Figure 1** Effects of wortmannin and LY294002 on the spontaneous quantal discharge. Isolated phrenic nerve-diaphragms were incubated in Tyrode solution. (A) Time-dependent actions of wortmannin and of LY294002 on the firing of miniature endplate potentials (mepps). (B) mepp amplitude distribution histograms before or 20–30 min after treatment with wortmannin. (C) Traces of mepps from different endplates of the same preparation in the absence (top panel), or treated with wortmannin 30 $\mu$M for 20 min (middle), or for 220 min (bottom). Resting membrane potentials were −76 ~ −82 mV. Data in (A and B) were pooled from 21–32 endplates, $n=4$–6.

vs $1.7 \pm 0.3$ mV, Figure 2A and B) but mepp frequency increased substantially to 35–60 events/s. The increase of mepp frequency persisted at least 5 h. Application of wortmannin (30–100 $\mu$M) progressively depressed the high level quantal discharge to $<3$ events/s in 2 h (Figure 2A and B). When the preparation was pretreated with wortmannin, subsequent incubation with high KC1 solution elicited a limited increase of quantal discharge ($<5$ events/s, Figure 2C). By contrast, after washout of wortmannin, high KCl solution elicited a significant increase of mepp frequency to 10–20 events/s (Figure 2B and C).

### *Inhibition of α-latrotoxin-induced mepp burst*

It has long been known that α-latrotoxin can evoke exocytosis independent of extracellular ion influxes and $Ca^{2+}$ release from intraterminal stores (Rosenthal *et al*., 1990; Lang *et al*., 1998). We examined the effects of wortmannin on this mode of neurotransmitter release. Shortly after ($\sim$10–20 min) bath application of α-latrotoxin (1–3 nM), nerve terminals intermittently generated mepp bursts, which often exceeded 500 firings/s and lasted 2–10 s (Figure 3A). For each endplate examined, 2–7 bursts were sampled within the 5 min observation period. The membrane around the endplate area depolarized by $\sim$5 mV during bursts ($-75.9 \pm 2.2$ vs $-81.6 \pm 1.9$ mV, $n=5$, 28 endplates), while restored to control level during dormant period ($-79.4 \pm 2.1$ mV). Burst episodes recurred for 5 h, though at 4–5 h the burst intensity decreased (burst episode reduced to 2–3 bursts/5 min, burst duration shortened to 2–5 s, and discharges within burst decreased to 50–200 events/s). Even after long-term treatment with α-latrotoxin, nerve stimulation (in the presence of $\mu$-conotoxin to block muscular $Na^+$ channels and twitches) still evoked epps, indicating that the releasable neurotransmitter pools were not yet exhausted. The epp amplitude, however, was reduced by $\sim$40% ($21.4 \pm 3.6$ vs $34.7 \pm 2.8$ mV, $n=5$, 25 endplates).

Wortmannin (10–100 $\mu$M), in addition to depress non-burst quantal discharge, progressively reduced the toxin-induced mepp bursts. Within 40–80 min, depending on the concentration of wortmannin employed, massive quantal release was reduced to $<2$ bursts/5 min accompanied by shortened burst duration ($<2$ s/burst) and diminished quantal release within bursts ($<100$ events). After long-term treatment with wortmannin for 2–3 h bursts were not detected, as illustrated in Figure 3A. The inhibition time course and inhibition potency of wortmannin on mepp bursts were similar to those on KC1-triggered quantal releases. When the preparation was first treated with wortmannin to suppress quantal release,

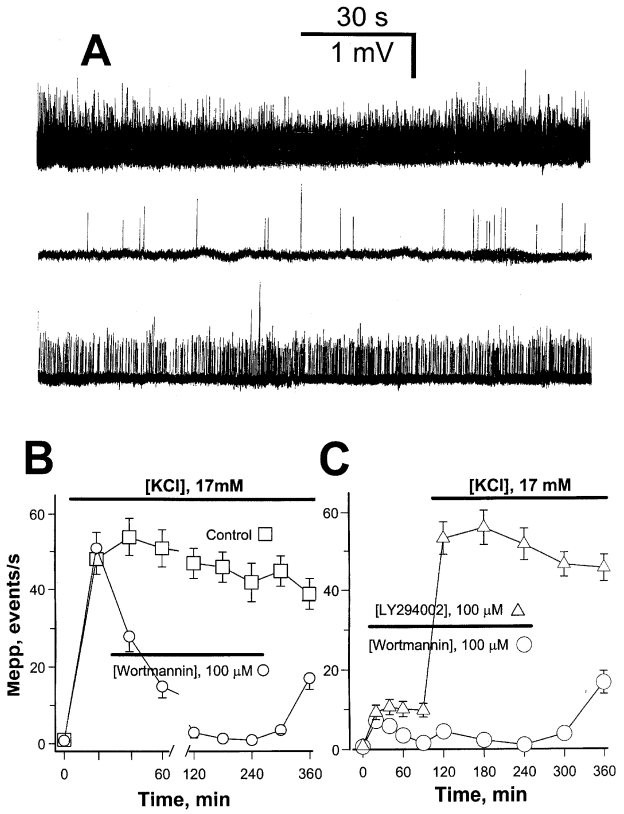


**Figure 2** Wortmannin, but not LY294002, inhibits quantal discharge evoked by high KC1 solution. (A) The preparation was incubated in high KC1 (17 mM) Tyrode solution for 20 min (top panel) and then further treated with wortmannin 100 $\mu$M for 220 min (middle). The bottom trace was 120 min after washout of wortmannin. Traces from different endplates. (B) Inhibition time course. Preparations were incubated in high KC1 solution in the absence or presence of wortmannin as indicated by the respective bars. The total incubation time for wortmannin was 220 min. (C) Prevention of high KC1 solution-evoked quantal release by wortmannin. Preparations were firstly treated with wortmannin or LY294002 for 90 min and then KC1 concentration was raised to 17 mM (total incubation time 270 min) as annotated. The total incubation duration for wortmannin and LY294002 was 240 min. Resting membrane potentials were $-60 \sim -64$ mV. Data in (B and C) were pooled from 22–27 endplates, $n=5$.

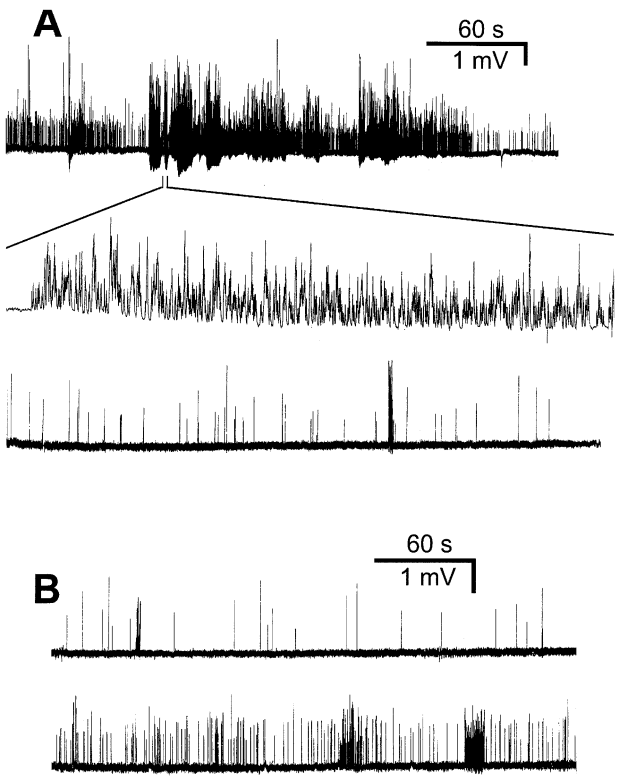


**Figure 3** Inhibition of α-latrotoxin-induced mepp burst by wortmannin. (A) The preparation was treated with α-latrotoxin 3 nM for 20 min (top panel), and 40 min later further with wortmannin 100 $\mu$M for 220 min (bottom, a different endplate). A part of mepp burst is displayed with a 200 fold expansion of the time scale (middle panel). (B) The preparation was first treated with wortmannin 100 $\mu$M for 220 min and then further with α-latrotoxin 10 nM for 40 min (top panel). Bottom panel shows a segment of recording continued from top panel 120 min after washout of α-latrotoxin and wortmannin. Note the significant increase of mepps and partial restoration of mepp burst. Resting membrane potentials were $-70 \sim -82$ mV.

subsequent application of α-latrotoxin did not elicit burst discharges (Figure 3B, top panel). Washout of both wortmannin and α-latrotoxin partially restored mepp bursts. In $Ca^{2+}$ -free Tyrode solution, α-latrotoxin also evoked mepp bursts. This toxin-induced intensive release was inhibited if the preparation had been pretreated with wortmannin (not shown).

*Effects of wortmannin on epps*

The above results indicate that wortmannin depressed KC1- or α-latrotoxin-evoked intensive asynchronous quantal release. The effects of wortmannin on nerve stimulation-evoked phasic quantal release were also explored. Contrary to the marked initial stimulatory effect on the spontaneous quantal discharge, wortmannin produced a significant inhibition of epps (evoked at 0.3 Hz) within 10 min of application (Figure 4). The onset of inhibitory action on epps preceded that on spontaneous discharge by ~30 min. The epp amplitude 4 h after treatment with wortmannin at 10, 30 and 100 μM was depressed by ~50, 85 and 95%, respectively. Because the mepp amplitude was unchanged and there were no significant alterations in epp rise times ($0.9\pm0.2$ vs $0.7\pm0.2$ ms, $n=5$, 24–29 endplates) and epp durations ($3.8\pm0.4$ vs $3.3\pm0.3$ ms), it seems that the quantal content of epps, estimated by the direct method, decreased in parallel to the extent of epp inhibition. Intensive nerve stimulations (trains of pulses at 50 Hz for 2 s every 30 s) did not accelerate the rate of inhibition. The epp amplitude was restored partially after washout of wortmannin (Figure 4). At high concentration (300 μM), wortmannin depressed the amplitude of compound nerve action potentials and caused abrupt failure of epps (rather than graded depression). In view of an inhibitory effect of wortmannin on muscle $Na^+$ channel (Hong & Chang, 1998), these results suggest that at high concentration the agent could interrupt impulse conduction of the phrenic nerve.

Since the phrenic nerve discharges at high rates under physiological conditions, the effects of wortmannin on evoked quantal release was studied further by analyses of the profile of epps under high frequency (50–100 Hz) nerve stimulation. In control, the epps from every endplate ran down consistently to a steady state, being 70–80% that of the first epp. After treatment with wortmannin (30–100 μM) for 40–240 min when epp amplitude was depressed down to 5–10 mV (i.e., 15–30% of control level), the profile of epps exhibited run-up (16 out of 37 endplates, for definitions see Methods), flat (11/37) or run-down (10/37) (Figure 5). The overall epp amplitude at steady state was $151\pm12$% relative to the first one. Statistical analyses revealed that during high frequency stimulation wortmannin increased the coefficient of variance of epps ($0.211\pm0.026$ vs $0.041\pm0.011$, pooled from 27–37 endplates, $n=5$–6). Depression of epp amplitude and increase of the coefficient of variance of epps are indications of reduced quantal release, which probably results from decreased $Ca^{2+}$ influx. At the neuromuscular junction, inhibition of $Ca^{2+}$ influx tend to induce a run-up profile of synaptic transmissions during high frequency stimulation (Magleby, 1973). We therefore compared the effects of wortmannin with those induced by ω-agatoxin IVA, which inhibits depolarization-induced, but not α-latrotoxin-induced, quantal release by selective blockade of P/Q type $Ca^{2+}$ channels on the motor nerve terminal (Hong & Chang, 1995). ω-Agatoxin IVA (5–10 nM) depressed epp amplitudes to 5–10 mV in 20–40 min without increasing spontaneous quantal discharge. Although the degree of the inhibition of epp was similar to that produced by wortmannin, the epps on high frequency nerve stimulation always displayed run-up with respect to the first one (up to $235\pm26$%, $n=4$, 19 endplates, Figure 5E), qualitatively and quantitatively different from those treated with wortmannin.

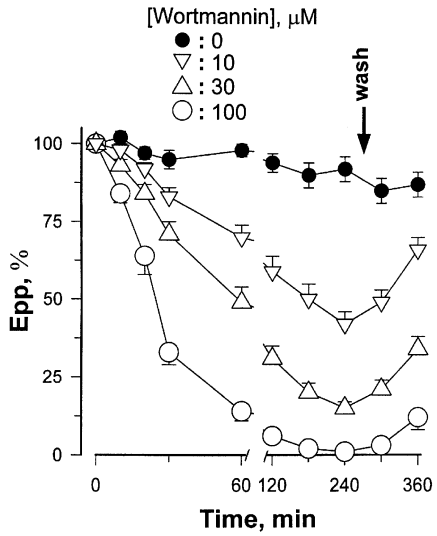


**Figure 4** Dose- and time-dependent actions of wortmannin on endplate potentials. Phrenic nerve was stimulated at 0.3 Hz, and 20 endplate potentials (epps) were averaged for each endplate. Wortmannin was washed out at 250 min. Data were pooled from 21–30 endplates, $n=4$–6.

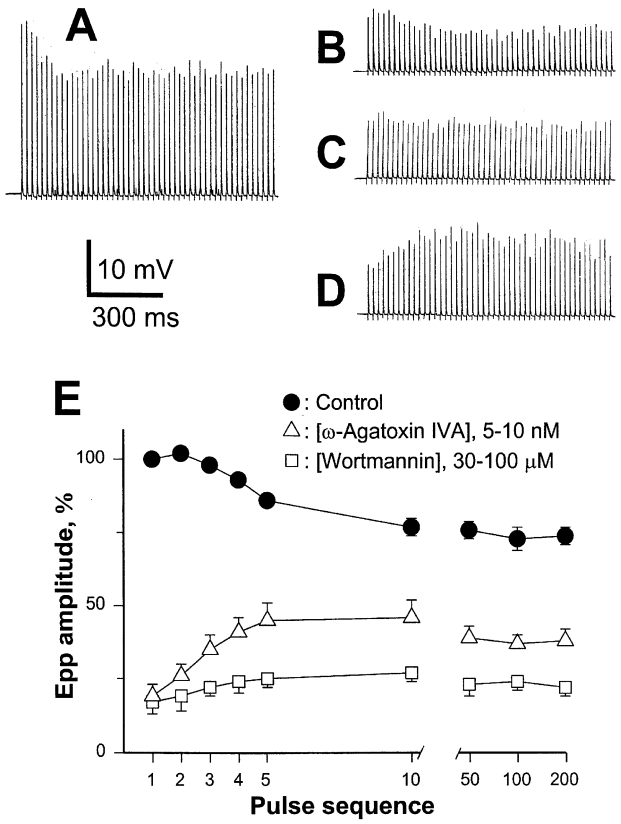


**Figure 5** Effects of wortmannin on epps evoked by high frequency stimulation. Phrenic nerve was stimulated at 50 Hz for 4 s in the absence (A), or presence of wortmannin 100 μM treated for 30–40 min (B–D). Shown are typical patterns of epps with run-down (B), flat (C) or run-up (D). (E) is the overall statistical epp profile of control, after treatment with wortmannin for 30–180 min, or after treatment with ω-agatoxin IVA for 20–40 min. Data were pooled from 19–37 endplates, ($n=4$–6). Resting membrane potentials were −76 ~ −82 mV.

These results suggest that the suppression of phasic quantal release by wortmannin was not due entirely to a blockade of $Ca^{2+}$ channel.

### *Effects of LY294002*

Due to the tiny structure of the nerve terminal, it is rather difficult to assay the influence of wortmannin on the activity of intraterminal kinase. The role of kinases in transmitter release was estimated pharmacologically by comparing the effects of wortmannin with those of LY294002, another membrane permeable inhibitor of PI 3-kinase (Vlahos *et al*., 1994). At concentration range of 10–100 $\mu$M, LY294002 elevated mepp frequency to 5~10 events/s. The increase lasted for 5 h (Figure 1A). Spontaneous discharges with long rise time and large amplitude occurred frequently (not shown). When treated with high concentration of LY294002 (100 $\mu$M), junctional as well as extra-junctional membranes depolarized by ~10 mV ($-70.2\pm2.5$ vs $-82.3\pm1.7$ mV, $n=4$, 22 endplates). As muscle membrane depolarized, mepp amplitude decreased by ~30% ($1.2\pm0.2$ vs $1.7\pm0.3$ mV) and epp amplitude decreased by a similar extent ($24.3\pm2.1$ vs $35.7\pm1.8$ mV, $n=3$, 17 endplates). After correction and normalization, the epp amplitude ($32.5\pm1.9$ mV) was only slightly less than that of control, suggesting that the release of neurotransmitter on nerve stimulation was not greatly depressed. These effects of LY294002 were partially reversible. On high frequency nerve stimulation, the epps at steady state were $79\pm3$% relative to the first epp ($n=3$, 17 endplates) and the epp profile was similar to that of control. Unlike wortmannin, LY294002 did not inhibit the quantal discharge evoked by high KC1 solution (Figure 2C, c.f. Figure 2B for the control frequency increase in high KC1 medium) or by α-latrotoxin (not shown).

## Discussion

At the neuromuscular junction wortmannin suppressed depolarization-induced quantal discharge and increased the coefficient of variance of epps without alteration of the elementary quantal size. These results suggest that in the presence of wortmannin fewer quanta are recruited for triggered exocytosis. However, the profile of epps on high frequency stimulation was not invariably characterized by prominent facilitation, and was thus unlike the well-documented run-up phenomenon whenever the quantal release is depressed by blockade of nerve terminal $Ca^{2+}$ channel.

In addition to inhibiting $Ca^{2+}$-dependent quantal release, wortmannin also suppressed the quantal discharge provoked by α-latrotoxin. α-Latrotoxin stimulates exocytosis either in $Ca^{2+}$ -containing or $Ca^{2+}$-free medium (Misler & Hurlbut, 1979; Rosenthal & Meldolesi, 1989; Lang *et al*., 1998). Research data indicate that α-latrotoxin is translocated through/incorporated into presynaptic membrane. The toxin binds in a $Ca^{2+}$-dependent manner to neurexin, a neuron surface protein, and might complex with nerve membrane to form a cation ionophore (Rosenthal *et al*., 1990; Filippov *et al*., 1994; Missler & Südhof, 1998; Sugita *et al*., 1999). The influx of $Ca^{2+}$ through this non-selective channel undoubtedly contributes to the increased exocytosis. In $Ca^{2+}$-free medium, the toxin binds to a G-protein-coupled receptor, which interacts with syntaxin, one of the key synaptic proteins in membrane fusion (Krasnoperov *et al*., 1997). Results from molecular and genetic approaches suggest that the receptor might function as a conduit for directing the toxin to the unknown downstream site of action without transducing exocytosis (Ichtchenko *et al*., 1998; Sugita *et al*., 1999), or might serve as an inhibitory modulator for quantal secretion (Bittner *et al*., 1998). Roles of the endogenous ligand of this $Ca^{2+}$-independent receptor for α-latrotoxin await characterizations.

### *Mode of action of wortmannin*

The inhibition of nerve stimulation- as well as α-latrotoxin-evoked quantal release suggests that, at the neuromuscular junction, wortmannin could attack $Ca^{2+}$-regulated and $Ca^{2+}$-independent exocytosis pathways. Alternatively, reduced quantal discharge could be due indirectly to decreased transmitter depots. Histological examinations of presynaptic ultrastructure could unravel whether wortmannin depletes synaptic vesicles.

A well-known class of inhibitor targeting on exocytotic release is botulinum/tetanus toxin, which enzymatically excises specific vesicle membrane protein (synaptobrevin) and presynaptic membrane proteins (syntaxin and synaptosome associated protein of 25 kDa–SNAP25, see Jahn *et al*., 1995 for review). These proteins are essential to the fusion of vesicle with nerve membrane, and a dysfunction of any one of them retards exocytosis (Jahn *et al*., 1995; Robinson & Martin, 1998). Polypeptide toxin carrying intrinsic phospholipase $A_2$ activity, such as $\beta$-bungarotoxin, is another class. These neurotoxins suppress quantal release probably by $Ca^{2+}$-activated enzymatic destruction of the membrane architecture of the nerve terminal (Fletcher & Rosenberg, 1997). The quasi-irreversible nature and nerve activity-dependent inhibition by these presynaptic neurotoxins contrast with the stimulus-independent effect of wortmannin, suggesting that, if wortmannin acts on these steps, the action mechanism is different from the polypeptide neurotoxins.

Given that phosphoinositides and PI kinases play important roles in vesicle exocytosis and membrane traffic (Hay *et al*., 1995; De Camilli *et al*., 1996) and that wortmannin, by inhibition of PI kinases, retards exocytosis in neuroendocrine and lymphoid cells (Folli *et al*., 1997; Martin, 1997), wortmannin might target similar critical steps to inhibit quantal release in the motor nerve. The partial reversibility in the mouse neuromuscular junction might be due to tissue- and species-difference (*c.f*. Burke *et al*., 1996). However, since the concentrations of wortmannin used were greater than those claimed for specific inhibition of the enzymes, and LY294002 did not inhibit exocytosis, it remains to be elucidated whether the depression of quantal release in the nerve-muscle tissue is ascribed to the inhibition of PI kinases. In cerebral cortical synaptosome, although PI kinase actively regulates $Ca^{2+}$-dependent exocytosis, wortmannin does not inhibit glutamate release (Wiedemann *et al*., 1998).

Despite the inhibition of evoked release, wortmannin increased spontaneous quantal discharge–at least before this action was dominated by depression phase. Elevated mepp frequency could be due to increased $Ca^{2+}$ influx, which would be blunted by exclusion of extracellular $Ca^{2+}$, and/or release of intracellular $Ca^{2+}$, which would tend to facilitate evoked release also. However, the experimental data do not favour these possibilities. Impairment of evoked quantal release with simultaneous recruitment of spontaneous quantal discharge are characteristics of the synaptic transmission in synaptotagmin-deficit mutant of *Drosophila* (Broadie 1995; Littleton & Bellen, 1995). Synaptotagmin is a vesicle membrane associated protein, which could function as a fusion clamp to prevent spontaneous fusion and to control regulated exocytosis

(Martin *et al*., 1995). As the $Ca^{2+}$ channel of nerve terminal is activated, the $C_2$ domain of synaptotagmin binds with $Ca^{2+}$, which not only changes the specificity of phosphoinositide binding to synaptotagmin but also disables the inhibitory function of synaptotagmin (Schiavo *et al*., 1996; Ubach *et al*., 1998). These reactions together with the recruitments of cytosolic fusion/regulatory proteins enable the juxta-paired synaptobrevin-syntaxin-SNAP25 to catalyze $Ca^{2+}$-dependent exocytosis. (Fernández-Chacón & Südhof, 1999). It is possible that wortmannin might alter the functions of synaptotagmin and other intraterminal proteins to differently affect spontaneous exocytosis and $Ca^{2+}$-triggered multiquantal release. Because both wortmannin and LY294002 elevated mepp frequency, further experiments could verify whether PI kinases modulate spontaneous quantal discharge.

In conclusion, aside from possible depression of $Ca^{2+}$ influx and interference of phosphoinositide metabolism, wortmannin impairs neurotransmitter release from motor nerve probably *via* modification of exocytotic cascade.

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