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# Inhibition of mouse neuromuscular transmission and contractile function by okadaic acid and cantharidin

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**1** Phosphorylations of cellular proteins modulate biological activities. The effects of okadaic acid (0.1–10 $\mu$M) and cantharidin (1–100 $\mu$M), inhibitors of protein phosphatases, on the synaptic transmission at the mouse neuromuscular junction were explored.

**2** Both inhibitors almost completely depressed twitch forces elicited by electrical stimulation of diaphragm muscles (the $IC_{50}$s for okadaic acid and cantharidin were $1.1 \pm 0.2$ and $13 \pm 1$ $\mu$M, $n = 5$, respectively) and suppressed contractures evoked by high $K^+$ and ryanodine more than 70%. Contractures caused by cardiotoxin, which destroys the integrity of sarcolemma, were not depressed.

**3** Both okadaic acid (10 $\mu$M) and cantharidin (100 $\mu$M) depolarized muscle membranes from $\sim -80$ to $\sim -60$ mV in a partially reversible and tetrodotoxin-sensitive manner. The initial short-term enhancement of twitch responses (up to $\sim$40%) was correlated with the inhibitors-induced repetitive firings of muscle action potential.

**4** Treatment with either agent resulted in nearly complete inhibitions of endplate potential (epp). The $IC_{50}$s were $0.8 \pm 0.2$ and $9 \pm 2$ $\mu$M ($n = 5$), respectively, for okadaic acid and cantharidin. On high frequency stimulation, the coefficient of epps was increased more than 10 fold and the extent of epp run-down during stimulations intensified from $\sim$25 to $\sim$75%. Analyses of presynaptic quantal releases revealed decreases in epp quantal content and the immediately available vesicle pool.

**5** The frequency of miniature epp was initially elevated up to 2 fold then suppressed down to $\sim$30%. The small reduction in the amplitude was antagonized when the membrane of endplate area was repolarized.

**6** The data suggest that okadaic acid and cantharidin inhibit mobilizations of synaptic vesicles and depress $Ca^{2+}$ release from sarcoplasmic reticulum and that protein phosphatases participate in the modulation of motor function.

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**Keywords:** Cantharidin; okadaic acid; ryanodine; endplate potential; protein phosphatase inhibitor

**Abbreviations:** epp, endplate potential; mepp, miniature endplate potential; OA, okadaic acid

## Introduction

Many lines of evidence suggest that phosphorylation and dephosphorylation of specific proteins modulate diverse aspects of cellular activities. For instance (though not unanimously), the efficacy of synaptic transmission is tuned by the phosphorylation state of the nerve terminal (Walaas & Greengard, 1991; Mulkey *et al*., 1993; Turner *et al*., 1999); the activation of $Ca^{2+}$ channel is facilitated by phosphorylation (Artalejo *et al*., 1992; Groschner *et al*., 1996); phosphorylation of myoplasmic proteins alters the contractile characteristics of skeletal muscle (Sweeney *et al*., 1993). As protein phosphorylation or dephosphorylation is dynamically processed by cytosolic kinases and phosphatases intensive stimulation/inhibition of the enzymes would be anticipated to alter integrated homeostasis (Ämmälä *et al*., 1994; Herzig & Neumann, 2000). There is extensive documentation correlating protein kinases and cellular functions whereas relatively sparse documentation is focused on the roles effected by phosphatases.

Okadaic acid (OA), a polyether fatty acid produced by dinoflagellates responsible for diarrhetic shellfish poisoning, has been shown to inhibit serine/threonine protein phosphatases 1 and 2A (Bialojan & Takai, 1988; Cohen *et al*., 1990). These phosphatases are widely distributed in the body of animals including the area of synaptic junction (Shields *et al*., 1985; Dosemeci & Reese, 1993). In the neuromuscular junction of a frog, OA reversibly enhanced neurotransmitter release with or without changes in the membrane potential and input resistance (Abdul-Ghani *et al*., 1991; Swain *et al*., 1991). However, increases in spontaneous quantal release and quantal size without effects on quantal content were also reported (Arenson & Gill, 1996). In this paper, the influences of OA and cantharidin, an anhydride polyring protein phosphatase inhibitor produced by blister beetles (Honkanen, 1993), on the synaptic transmission of mammalian neuromuscular junction were explored. The two compounds produced similar unique pharmacological effects, and the results suggest modulatory roles of phosphatases in the synaptic transmission.

## Methods

Phrenic nerve-hemidiaphragms were isolated from mice (ICR strain, 20–25 g). The nerve-muscle preparations were bathed in Tyrode solution (composition in mM: NaCl 137, KCl 2.8, $CaCl_2$ 1.8, $MgCl_2$ 1.1, $NaH_2PO_4$ 0.33, $NaHCO_3$ 11.9 and glucose 11.2) maintained at 35–37°C with the pH adjusted at 7.3–7.4 by aeration with 5% $CO_2$ in $O_2$. Every nerve-muscle preparation was equilibrated for 1–1.5 h before experiments.

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*Contraction experiments*

Contractions were evoked by stimulation of the nerve trunk with supramaximal rectangular pulses of 0.03 ms duration (indirect stimulation) or by field stimulation of diaphragm muscles with pulses of 0.3 ms duration (direct stimulation). Before any treatment, the twitch force of test group was 95–105% with respect to the related control pair. The direct and indirect stimulations were terminated 5 min before the challenge of contracture-inducer to minimize possible activity-dependent effect. Contractures of hemidiaphragm were monitored for 35 min to cover peak/plateau response. Contractile responses were recorded isometrically and quantified by amplitude (for twitches) or by contraction-time integral (for contractures).

*Electrophysiological studies*

Glass microelectrodes for intracellular recording were filled with 3 M KCl and had a resistance of 3–10 MΩ. Muscle action potentials were evoked with depolarizing current (20–80 nA for 5 ms) injected *via* the recording electrode using a single electrode current clamp amplifier (Dagan 8100, Minneapolis, U.S.A.). Prior to the injection of depolarizing current, the muscle fibre was hyperpolarized by cathodal current (ranging from 7.5–50 nA for 300 ms which set the local membrane potential at −75 to −85 mV) to reduce the inactivation of muscle $Na^+$ channel. The slopes of the resultant pre-conditioning current-voltage plots were used for estimations of membrane resistance.

Endplate potentials (epps) were recorded in preparations immobilized with $\mu$-conotoxin (1 $\mu$M). Protracted tracking of synaptic responses from the same endplate were performed by recording synaptic currents extracellularly to avoid depolarization drifts of membrane potential that arose during a long-term intracellular impalement with microelectrode. Quantal content was estimated from the quotient of the mean of epp (or endplate current) over the mean of mepp (or miniature endplate current), the synaptic responses being sampled within the same 1 min period. Compound nerve action potentials were recorded extracellularly with a glass microelectrode (filled with 1 M NaCl, resistance 10–20 MΩ) pierced through the encapsulating sheath of nerve trunk.

Membrane potentials and epps were d.c.-coupled whereas high gains of compound nerve potentials, miniature epps (mepps) and synaptic currents were recorded in a.c. mode. Waveforms were digitized and displayed on a thermal recorder (Gould TA5000-AM800, Cleveland, OH, U.S.A.). Epp amplitudes were corrected for non-linear summation, assuming 0 mV for the reversal potential, and then normalized to a reference membrane potential of −80 mV (*c.f.* Hong & Chang, 1999).

*Statistics*

Epp amplitude was the mean from 4–7 different endplates each with 20 epps evoked at 0.3 Hz. Mepps with amplitude larger than 4 mV usually were characterized with a slow rise time (1–3 ms) and long duration (4–10 ms), greatly dissimilar to the contour of normal mepps (<0.8 ms rise time, 2.8–3.5 ms duration). These giant mepps, probably reflecting decreases in the interaction of individual components or quantal releases from outside the active zones, occurred at a rather low probability (~1 event/1–3 min) and were not included in the estimation of quantal size. Each set of experiments consisted of 3–6 preparations and the pooled statistical data were expressed as means±s.e.mean. Differences between means were analysed by Student's *t*-test and a statistical *P* value of less than 0.05 was considered significant.

*Chemicals*

$\mu$-Conotoxin GIIIB (Bachem Feinchemikalien AG, Bubendorf, Switzerland), veratridine (Sigma Chem. Co., St Louis, MO, U.S.A.), okadaic acid sodium and ryanodine (Research Biochemicals International, Natick, U.S.A.) were all dissolved in Tyrode solution. Cantharidin (Biomol, Plymouth Meeting, U.S.A.) was dissolved in dimethylsulphoxide. The solvent in the organ bath was kept <0.05% (v $v^{-1}$).

## Results

*Effects of OA and cantharidin on contractile responses*

The twitch responses evoked by alternate low frequency stimulation (at 0.1 Hz) of nerve and muscle maintained a steady value (20±2 mN, $n=5$) at least for 3 h in control. The force of tetanic contractions evoked on high frequency stimulation (100 Hz for 5 s) summated to a plateau (209±11 mN) approximately 10 times that of twitch force. After treatment with a high concentration of OA (3–10 $\mu$M) or cantharidin (30–100 $\mu$M), twitch forces on indirect or direct stimulations were augmented up to 39±4% ($n=5$, in this and the following cases, if the data of OA and cantharidin were pooled, the *n* number indicates that either agent was tested for *n* preparations, but not that both agents were tested for a total of *n* preparations) over the control level for the initial 3–15 min (Figure 1A). Tetanic contractions, however, were not enhanced (1012±26 vs 1039±21 smN). During the augmentation phase, muscle tone was elevated slightly over the resting

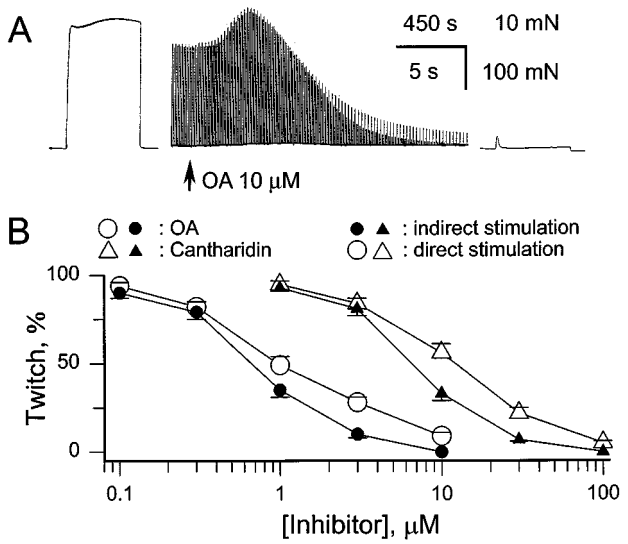


**Figure 1** Effects of okadaic acid (OA) and cantharidin on the contractility of mouse diaphragm. (A) Biphasic effects of OA on twitch forces. Left panel: tetanic contraction evoked by direct stimulation at 100 Hz for 5 s. Middle: twitches were elicited by alternate direct and indirect stimulations at 0.1 Hz. The larger responses in each couple are twitches on direct stimulation. Right panel: tetanic contraction 120 min after treatment with OA. The breaks between panels indicate 10 fold changes in the gain of contractility. (B) Concentration-dependent inhibitions of twitch responses in diaphragms treated with OA or cantharidin for 90 min. Data (mean±s.e.mean) pooled from five different preparations.

tension (up to $0.9 \pm 0.2$ mN) and contraction duration prolonged (90% contraction duration: $36 \pm 3$ vs $32 \pm 2$ ms, $P < 0.05$, paired $t$-test, $n = 5$). Thereafter, twitches and tetanii were depressed and washout of OA and cantharidin did not restore contractile responses (not shown). At low concentration ranges (0.3–1 $\mu$M for OA, 3–10 $\mu$M for cantharidin), both agents depressed twitch forces progressively without producing twitch enhancements. The $IC_{50}$s estimated from the effect on twitches elicited by direct stimulation at 90 min treatment with OA and cantharidin was $1.1 \pm 0.2$ and $13 \pm 1$ $\mu$M ($n = 5$), respectively (Figure 1B). The twitches evoked by indirect stimulation were more vulnerable than those evoked by direct stimulation.

Increases in twitch force could be resulted from reduced sequestration of myoplasmic $Ca^{2+}$ and/or increased $Ca^{2+}$ release from cellular stores. These possibilities, however, are incompatible with the finding that OA and cantharidin did not augment tetanic contraction-time integral even during the period that twitch responses were augmented. For a sharp contrast, thapsigargin (1–3 $\mu$M), an inhibitor of sarcoplasmic reticulum $Ca^{2+}$ pump, enhanced the amplitude and prolonged the duration of tetanii to $217 \pm 22$ and $1029 \pm 68$% ($n = 4$), respectively. In preparations pretreated with veratridine (0.1–0.3 $\mu$M), which induced repetitive firings of $Na^+$ spike and thereby potentiated twitch force (up to $96 \pm 18$% over control, $n = 4$), OA and cantharidin failed to cause further augmentations (the twitches at maximum were $104 \pm 3$% of those treated with veratridine alone). The results described in the later experiments revealed that the two phosphatase inhibitors induced repetitive firings of muscle action potential.

### *Inhibition of KCl- and ryanodine-induced contractures*

In order to locate the possible site of action that leads to impairments of contractile responses, effects of OA and cantharidin on contractures induced by high $K^+$, by ryanodine, or by cardiotoxin were explored. High $K^+$ solution, by causing persistent depolarizations of muscle membrane, produces contractures *via* sustained stimulations of the routine excitation-contraction coupling pathway. Ryanodine, by contrast, skips the electrical activation step and directly activates the $Ca^{2+}$ release channels on the sarcoplasmic reticulum to elicit contractures. The rationale for studying the effect on cardiotoxin-induced contracture is that, after destructions of the integrity of sarcolemma by cardiotoxin (Chang, 1979), the contractile system and the associated regulatory proteins are directly exposed to Tyrode solution (a medium enriched with unbound $Ca^{2+}$). Hence, the toxin provokes a contracture by-passing the gating of and $Ca^{2+}$ release from sarcoplasmic reticulum. Therefore, any effect on the contracture would have resulted from alterations in the contractile apparatus.

Challenge of high KCl Tyrode solution (50–100 mM) elicited a tetrodotoxin-sensitive phasic contraction and relaxation followed by tetrodotoxin-resistant tonic contractures (up to $63 \pm 5$ mN, $n = 4$, ~3 fold direct twitch force, Figure 2A). Ryanodine (3–100 $\mu$M) evoked tetrodotoxin-resistant contractures and the maximal elevation of muscle tone reached $107 \pm 6$ mN ($n = 5$, Figure 3A). The tonic contractures after high KCl solution or ryanodine decayed rather slowly over 60 min. When pretreated with OA or cantharidin for 1–2 h to ensure that twitch forces were depressed to <10% of control, both the magnitude and duration of high $K^+$/ryanodine contractures were strongly curtailed (Figures 2B and 3B,C). In contrast, OA and cantharidin did not depress small contractures caused by a

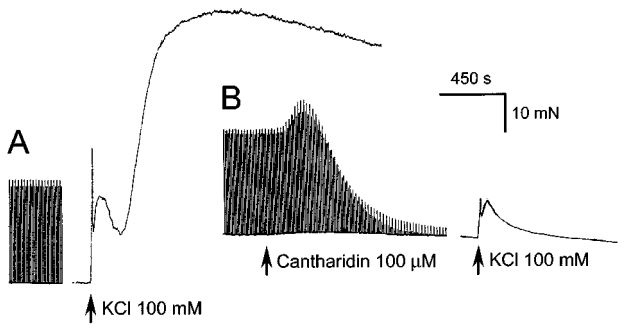


**Figure 2** Biphasic effects on twitch force and inhibition of $K^+$-contracture by cantharidin. (A) Twitches and $K^+$-contracture in control preparation. In this and the following figures, direct and indirect stimulations were terminated 5 min before the challenge of contracture inducer. (B) Another preparation, high KCl Tyrode solution was applied after twitches were depressed to <10% by cantharidin (pretreated for 60 min).

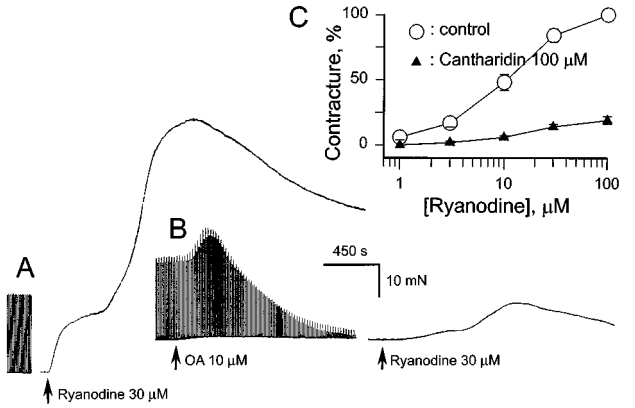


**Figure 3** Inhibition of ryanodine-contracture by OA and cantharidin. Ryanodine-contracture was induced in control (A), in preparations pretreated with OA (B), or with cantharidin (C) for 60 min. The ordinate in (C) denotes per cent contracture-time integral (each data point pooled from five different experiments), and the integral produced by 30 min treatment with ryanodine 100 $\mu$M was arbitrarily set 100%.

low concentration of cardiotoxin (0.3 $\mu$M, Figure 4). In nominal $Ca^{2+}$ free/high $Mg^{2+}$ (4.4 mM) Tyrode solution, the elevation of muscle tone in response to the challenge of cardiotoxin was strongly attenuated ($47 \pm 9$ vs $3310 \pm 63$ smN, $n = 4$), confirming that extracellular $Ca^{2+}$, rather than intracellular $Ca^{2+}$ stores, is mainly responsible for the toxin-induced contractures. These results demonstrate that the phosphatase inhibitors suppressed the contractile responses dependent on the $Ca^{2+}$ release from intracellular stores.

### *OA and cantharidin depolarize muscle membrane*

On application of either agent at a high concentration (OA: 10 $\mu$M; cantharidin: 100 $\mu$M), muscle membrane depolarized from the control level of $-81 \pm 2$ mV down to $-59 \pm 3$ mV ($n = 5$, Figure 5), and membrane resistance decreased ~10% ($604 \pm 23$ vs $688 \pm 29$ KΩ). At the depolarized state, the maximal rate of rise of muscle action potential decreased ~50% ($359 \pm 19$ vs $745 \pm 33$ V $s^{-1}$, $n = 5$), but the value was restored to $692 \pm 29$ V $s^{-1}$ when membranes were repolarized to $-75 \sim -80$ mV by current injections. There was no difference in the extent of depolarizations between junctional

area and extrajunctional site. Tetrodotoxin substantially but incompletely antagonized the membrane depolarizations (Figure 5). Similar to tetrodotoxin, $\mu$-conotoxin (1 $\mu$M) incompletely repolarized the resting membrane in preparations treated with cantharidin (100 $\mu$M, $-70\pm3$ vs $-58\pm4$ mV, $n=4$). Washout of OA and cantharidin restored membrane potential to a level close to that after blockades of muscle $Na^+$ channel (Figure 5) and the two manoeuvres did not yield an additive effect (after washout of the phosphatase inhibitor with Tyrode solution containing tetrodotoxin, the membrane potential was $-73\pm3$ mV, $n=4$). These results suggest that part of the induced depolarizations was mediated by a reversible activation of muscle $Na^+$ channel. In the presence of a high concentration of OA or cantharidin, repetitive firings of muscle action potential (up to 30 Hz) occurred either spontaneously or in response to injections of depolarizing current when the membrane was not yet depolarized beyond $-70$ mV (not shown). Both agents did not depress high $K^+$-induced membrane depolarizations ($-36.7\pm1.9$ vs $-39.2\pm2.0$ mV, $n=4$, in 50 mM KCl Tyrode solution), suggesting that the blockade of KCl-contracture was due to inhibitory effects beyond the step of sarcolemma depolarizations.

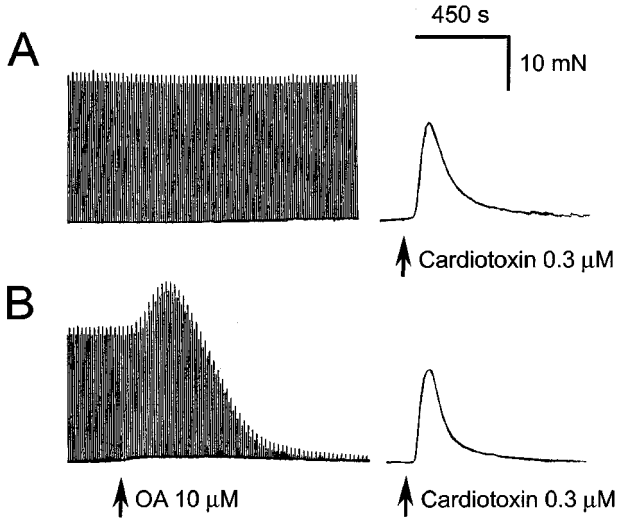


**Figure 4** OA did not depress contracture evoked by cardiotoxin. (A) Control twitch responses and contractures elicited by cardiotoxin, a protein that disrupt the integrity of sarcolemma. (B) Another preparation. Cardiotoxin was applied after twitches were depressed to $<10\%$ by OA (pretreated for 60 min).

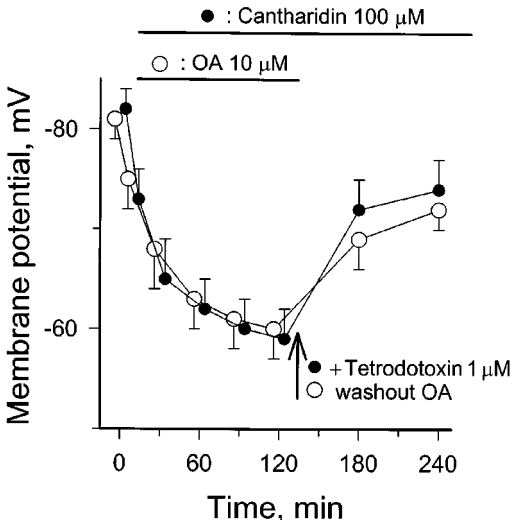


**Figure 5** OA and cantharidin depolarized muscle membrane. Preparations were treated either with OA or cantharidin for 120 min. Thereafter, at the arrow mark, OA was washed out or tetrodotoxin was added to the preparations already incubated with cantharidin. Data pooled from five experiments. Note the partial restorations of membrane potential after washout or treatment with tetrodotoxin.

### *Effects on mepp amplitude and frequency*

In control, mepp frequency increased slightly $\sim4-5$ h after isolation of the phrenic nerve-muscle tissue. It had been proposed that denervation/nerve transection might induce early depolarizations of motor nerve terminals and increase mepp frequency (*c.f.* Albuquerque *et al.*, 1971). OA and cantharidin produced a marginal increase of spontaneous quantal release during the initial 1–2 h ($2.3\pm0.3$ vs $1.1\pm0.2$ events $s^{-1}$, $n=5$, Figure 6A) and for the subsequent 2–4 h suppressed mepp frequency to $\sim30\%$ of the corresponding control ($0.9\pm0.2$ vs $2.4\pm0.3$ events $s^{-1}$). The time course of mepp was not altered ($3.2\pm0.2$ vs $3.1\pm0.2$ ms). As the membrane depolarized, mepp amplitude decreased moderately ($1.2\pm0.2$ vs $1.8\pm0.2$ mV, $n=5$). When the membrane of endplate area was repolarized to $-75\sim-80$ mV, mepp amplitude was restored to $1.6\pm0.2$ mV close to the control level, indicating that the endplate acetylcholine receptors are unaffected by the two agents. To further analyse the effects on mepp frequency, quantal discharges were intentionally elevated by high KCl Tyrode solution (15–20 mM) or by addition of veratridine (3–5 $\mu$M). Either treatment depolarized muscle membrane to $-55\sim-65$ mV (with a concomitant depression of mepp amplitude to $1.1\pm0.5$ mV, $n=4$) and

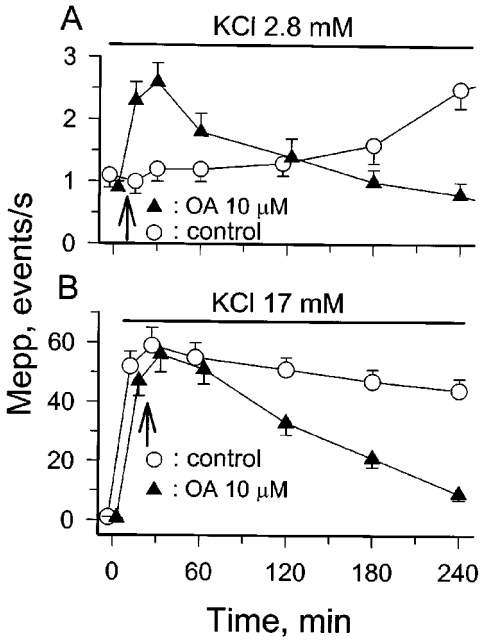


**Figure 6** Effects of OA on miniature endplate potential (mepp). (A) In preparations bathed in normal Tyrode solution (2.8 mM KCl), OA was added at $t=5$ min, causing an initial facilitation, then suppression, of spontaneous quantal release. Data pooled from five preparations. (B) Elevating KCl concentration to 17 mM (at $t=5$ min) increased mepp frequency. OA, applied at $t=25$ min, progressively reversed this increase. Data pooled from five experiments.

produced 20–60 fold increases of mepp-like responses (40–60 vs <2 event $s^{-1}$, Figure 6B). OA and cantharidin progressively suppressed the quantal discharges down to 15–40 events $s^{-1}$ (~50% of control) in 2 h and further to <10 events $s^{-1}$ after 4 h of treatment.

### *Effects on epps and quantal content*

The synaptic activities were studied in preparations paralyzed with $\mu$-conotoxin, which partially antagonized OA- and cantharidin-induced membrane depolarizations. Thus, the immobilization protocol offered an advantage that, if necessary, the muscle membrane could be hyperpolarized with fewer amounts of cathodal currents. Because the amount of neurotransmitter released by a given action potential can be modified by the previous activities of the release process, the phrenic nerve was excited using two stimulation paradigms. One at a low rate of stimulation (0.3 Hz) to minimize complications (facilitation or depression) on synchronized multiquantal releases, the other with high frequency of pulses (50–100 Hz) to highlight the role of transmitter mobilizations (described in the next section). Control experiments indicate that, under low frequency stimulations, amplitudes of epp or endplate current maintained at a steady level for 4–5 h. The effects on evoked transmitter release were assessed in two aspects. (1) Epps were sampled from the endplate areas that were periodically repolarized to −75 to −82 mV (for a period of 60 s every 3 min) to offset the phosphatase inhibitor-induced membrane depolarizations. OA (3–10 $\mu$M) and cantharidin (30–100 $\mu$M) increased epp amplitude slightly for the initial 10 min, the maximal value being 107±2% ($n=5$, paired) of control. Afterwards, epp amplitude decreased to <10% of control. Washout did not produce significant restorations of epp amplitude (Figure 7A). The $IC_{50}$s estimated at 90 min treatment with OA (0.1–10 $\mu$M) and cantharidin (1–100 $\mu$M) was 0.8±0.2 and 9±2 $\mu$M ($n=5$), respectively. (2) Instead of direct comparing changes in the postsynaptic responses, the presynaptic quantal release, a distinct event uninfluenced by depolarizations of the postsynaptic side, was measured. Quantal content, estimated from the synaptic currents recorded extracellularly, was monitored from the same endplate for 4 h. The result was similar to that measuring epp amplitude: both agents produced a short-lived slight enhancement of quantal content during the initial 10 min (for OA 3–10 $\mu$M: 38±4 vs 32±3, $n=5$, paired; for cantharidin 30–100 $\mu$M: 35±2 vs 30±2, $n=5$, paired) followed by a marked depression to <5 in 3–4 h. The inhibitions of epps and endplate currents were graded; abrupt/sudden failures of synaptic activities, signs associated with blockade of nerve impulse conduction, were infrequently observed. When treated with a high concentration of OA (10 $\mu$M) or cantharidin (100 $\mu$M) the amplitude of compound nerve action potential was depressed to 86±3% ($n=4$) of control.

### *OA and cantharidin intensify epp run-down*

The results aforementioned indicate that both agents suppress phasic synaptic transmission. To explore whether the depression was caused by inhibitions of $Ca^{2+}$ influx into the nerve terminals, effects on the quantal releases were compared with those effected by $\omega$-agatoxin IVA, a selective blocker of the nerve terminal P/Q type $Ca^{2+}$ channels (Hong & Chang, 1995), under high frequency nerve stimulation condition. This mode of activation rapidly depletes the immediately available vesicle pool as reflected by the prompt decline in the amplitude of successive epps (Figure 7B). The epps leveled off ~200 ms after starting stimulations and the mean amplitude of the 20–100th epps decreased by 20–25%. The statistical profiles of trains of epps in preparations treated with $\omega$-agatoxin IVA, OA or cantharidin are illustrated in Figure 8, which summarizes per cent amplitude deviation of the steady-state epps (with respect to the first epp) vs the absolute amplitude of the first epp. A minus value in the vertical axis denotes run-down of epps whereas a positive value indicates run-up of epps. This parameter can be regarded as an input and output status of transmitter mobilization, the lower the value, the less the supply (vs release). As the epp amplitude was depressed by $\omega$-agatoxin IVA the extent of epp run-down diminished (Figure 8), and when epp amplitude was reduced by 60–70% by the toxin conspicuous run-up of epps ensued. Reduced depletion of transmitters resulting from suppressions of $Ca^{2+}$ influx and progressive recruitment of active zones during stimulations could account for the run-up phenomenon. By contrast, as OA and cantharidin depressed epp amplitude, the profile of epps, rather than displaying facilitations, exhibited marked run-down. The amplitude at the steady-state relative to the first one was depressed by 70–80% (Figures 7C and 8). The immediate available pool of vesicles, estimated as the

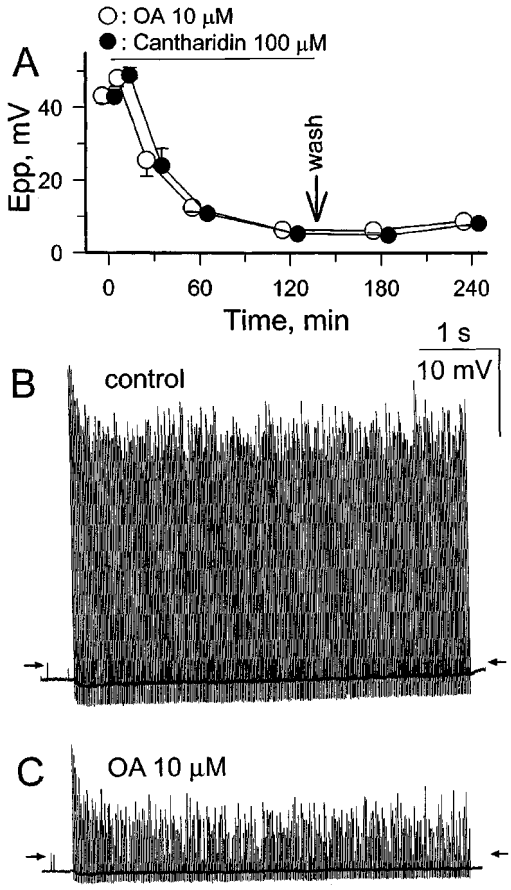


**Figure 7** Effects of OA and cantharidin on endplate potential (epp). Nerve-muscle preparations were immobilized with $\mu$-conotoxin. Epps were evoked with low frequency pulses (at 0.3 Hz, A) or with trains of high frequency stimulation (50 Hz for 5 s, B and C). OA or cantharidin was added at $t=5$ min and washed out at $t=135$ min (A, data pooled from five preparations). Trains of epps were obtained before (B) or after treatment with OA for 120 min (C). The resting membrane potential of the endplate area were repolarized to −78–−82 mV during samplings of epps and mepps. The randomly-occurred ticks (B, C) with amplitudes delimited between baseline and horizontal arrows were mepps. Note that OA increased the extent of epp run-down but left mepp amplitude undepressed.

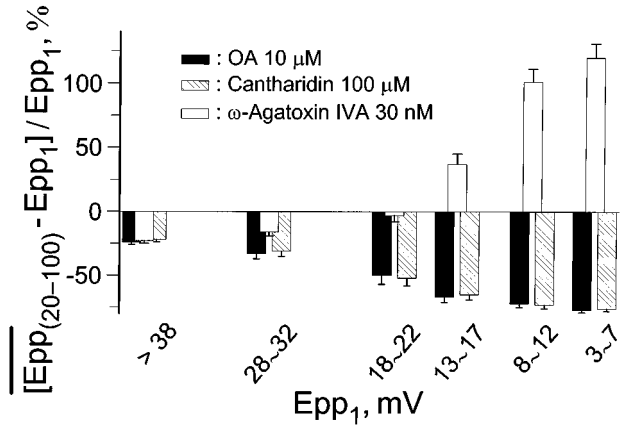


**Figure 8** Inhibitions of evoked quantal releases by $\omega$-agatoxin IVA, OA or cantharidin and the accompanied changes in epp profile. Quantal releases were evoked with trains of high frequency pulses (50–100 Hz for 5 s every 3 min). Abscissa is the absolute amplitude of the first epp ($Epp_1$) on a train of stimulation. The amplitudes are assorted in six categories and the group associated with the epp amplitude >38 mV depicts the control data (before any treatment). All the three agents depressed epp amplitude progressively down to 3–7 mV during 120 min experimental period. Ordinate is the per cent amplitude deviations of the $\overline{Epp}$ at steady-state, the mean amplitude of the 20–100th epps ($Epp_{(20-100)}$) during a train of stimulation, from the respective $Epp_1$. The parameter signifies epp profiles (minus sign for run-down of train of epps, positive sign for epp run-up) and reflects overall facilitation (treated with $\omega$-agatoxin IVA) or depression (with OA or cantharidin) of the mobilization of releasable transmitter quanta during intense stimulations. Data pooled from 5–6 preparations.

amount of quanta extrapolated from the sum of the initial 10 epps (*c.f.* Elmqvist & Quastel, 1965), was reduced to ~15% of control (95±8 vs 678±29, $n=5$). In the mean time, the amplitude fluctuation of individual epps within a train of stimulation increased (Figure 7C). The coefficient of variance 2 h after OA (3 $\mu$M) or cantharidin (30 $\mu$M) increased more than 10 fold (0.694±0.062 vs 0.039±0.009, $n=4$).

To examine whether the induced epp run-down could be caused by a use-dependent desensitization/blockade of the postsynaptic acetylcholine receptors, the mepp amplitude during the later parts (the 2–5 s time period) of train of high frequency stimulations was compared with those sampled without stimulations (in-between stimulation periods). Even treated with a high concentration of OA (10 $\mu$M) or cantharidin (100 $\mu$M), there was no statistical difference in mepp amplitudes between the two episodes (1.0±0.2 vs 1.2±0.2 mV, $n=4$, for the *in situ* depolarized endplate plates; 1.6±0.2 vs 1.7±0.3 mV, $n=4$, from endplates with the resting membrane repolarized to −78~−82 mV).

## Discussion

Both OA and cantharidin produced transient facilitations then caused sustained depressions of the contractile response as well as the neuromuscular transmission. The $IC_{50}$s of OA and cantharidin on the motor function were 10–100 fold higher than the respective values for the inhibition of cell-free protein phosphatases (Bialojan & Takai, 1988; Honkanen, 1993). This concentration gap between the effects on a tissue preparation and on a homogenate target can be seen in many instances. Because rather high concentrations were employed it might be argued that the effects were due to actions irrelevant to phosphatases. Although the activities of cytosolic phosphatases were not quantified, the following observations suggest that the actions of OA and cantharidin on the motor function could be linked to the inhibition of protein phosphatase(s) 1 and/or 2A. OA and cantharidin, in spite of bearing dissimilar chemical skeleton, exerted very similar pharmacological activities on the nerve-muscle preparation. Their inhibitory potency paralleled the rank order obtained from enzyme inhibition assays, OA being more potent than cantharidin in inhibiting the same subset of phosphatases.

### *Depression and facilitation of contractility*

The core step of the excitation-contraction coupling of skeletal muscle is assumed to be the transduction of t-tubule signals, the charge movements of the voltage sensor on the dihydropyridine receptor, to activate the $Ca^{2+}$ channel on the sarcoplasmic reticulum which is tonically inhibited by myoplasmic $Mg^{2+}$ and can be gated open by ryanodine (Franzini-Armstrong & Protasi, 1997). On appropriate depolarizations of sarcolemma, a cytosolic protein–FKBP12–is recruited to relieve $Mg^{2+}$-inhibition and to coordinate the opening of the intracellularly located $Ca^{2+}$ channels (Ahern *et al*., 1994; Stephenson *et al*., 1998). The findings that OA and cantharidin suppressed the contractures evoked by high $K^+$ and ryanodine, but not by cardiotoxin, reveal that the $Ca^{2+}$ release from sarcoplasmic reticulum, rather than the performance of contractile proteins *per se*, is suppressed. Phosphorylation of ryanodine receptor by endogenous or exogenous protein kinase produces multiple effects, ranging from facilitation, no effect to inhibition (Wang & Best, 1992; Hain *et al*., 1994). Since at all the concentrations tested OA and cantharidin inhibited contractile responses eventually, it seems that over phosphorylations of myoplasmic proteins (by inhibitions of phosphatases) impair the $Ca^{2+}$ release from myoplasmic stores. Whether the phosphatase inhibitors additionally suppressed transduction steps upstream to $Ca^{2+}$ release remains to be elucidated. Interestingly, OA exerts positive inotropic effects in human myocardium (Linck *et al*., 1996). The differential pharmacological effects on skeletal vs cardiac muscles are similar to those produced by wortmannin, an inhibitor of phosphatidylinositol kinase, and could be related to different modes of excitation-contraction couplings and different isoforms of ryanodine receptors in the two types of striated muscles (Hong & Chang, 1998).

The initial augmentation of contractile responses correlated temporally with OA- and cantharidin-induced repetitive dischargings of muscle action potential. It has been postulated that phosphorylations at distinct sites of muscle membrane could modulate membrane excitability and channel function (Bendahhou *et al*., 1995). In addition, phosphorylations of myosin light chain potentiate the contractile response of vertebrate striated muscles (Sweeney *et al*., 1993; Patel *et al*., 1998). It is inferred that repetitive activation and, to a less extent, increased efficiency of excitation-contraction coupling contribute to the enhancements of twitch force effected by inhibitions of protein phosphatase(s). Together, phosphorylation and dephosphorylation turnoverings of ion channels and intracellular regulatory proteins seem to comodulate the sequential transformations of electrical excitation, intracellular $Ca^{2+}$ mobilization and actomyosin response to shape mechanical events.

### *Inhibition of vesicle mobilization*

No clear effect of OA and cantharidin on the amplitude of elementary quantal response was observed. The quantal size

during high frequency stimulation was not changed either. These results indicate that the agents have little effect on the acetylcholinesterases and postsynaptic acetylcholine receptors. The effects on epps were thus attributed to presynaptic actions. In the rat neuromuscular junction and *Torpedo* electric organ, promotion of receptor phosphorylation enhances agonist-induced desensitizations of the nicotinic acetylcholine receptor (Albuquerque *et al*., 1986; Huganir *et al*., 1986). In the mouse endplate, phosphatase inhibitors perhaps do not cause significant desensitizations of the acetylcholine receptors.

Decreased quantal content on low frequency stimulation and increased coefficient of variance of epps during high frequency stimulation implicate that OA and cantharidin depressed $Ca^{2+}$-dependent neurotransmitter release. The inhibitory effect was accompanied by a conspicuous run-down of epps. This phenomenon (decreased transmitter release with intense epp run-down) is unique and is opposite to the general consensus that a run-up feature occurs after blockades of the nerve terminal $Ca^{2+}$ channel. Obviously, the depression of quantal release by the phosphatase inhibitors cannot be due simply to an inhibition of $Ca^{2+}$ influx. It has been hypothesized that synaptic vesicles are held in cluster/aggregate state by synapsins, a synaptic vesicle-associated protein, and appropriate phosphorylations of synapsins release the constraint making the vesicles available for release (Llinás *et al*., 1985; Greengard *et al*., 1993; Hosaka *et al*., 1999). A broad-spectrum inhibition of neuronal phosphatases by OA may deplete a subpopulation of synaptic vesicles to be recruited for $Ca^{2+}$-dependent exocytosis, presumably due to impairments of vesicle mobilization into or recycling from specific compartments (Vickroy *et al*., 1995; Issa *et al*., 1999). In the frog neuromuscular junction, OA disrupted vesicle clusters and interfered with the translocation of vesicles to presynaptic release sites, effects corresponding with the depressions of evoked release (Betz & Henkel, 1994). These results demonstrate an important role of dephosphorylation in preparing vesicles for release.

In the mammalian neuromuscular junction, nicotinic receptor antagonists induce enhanced run-down of epps/endplate currents on high frequency stimulation, suggesting the presence of autoreceptors on the nerve terminal. The run-down could be caused by inhibitions of the autoreceptor-mediated positive feedback (hence, resulting in diminished transmitter release and steady state epps, Bowman *et al*., 1990), or by removals of the autoreceptor-triggered negative feedback (hence, leading to augmented initial epp quantal content with consequent enlarged amplitude difference between the initial and the steady state epps, Domet *et al*., 1995). The inability of the nerve terminal to sustain a high release could be ascribed to insufficient mobilizations of transmitter quanta. It would be of interest to see whether presynaptic nicotinic receptor couples nerve terminal phosphatases/phosphokinases.

### *Modulation of transmitter release*

Neuronal phosphatases, however, might exert differential control over transmitter release. In central neural tissues, inhibitions of phosphatases enhance synaptic transmission and facilitate transmitter efflux (Sim *et al*., 1993; Herron & Malenka, 1994; Vickroy *et al*., 1995). In peripheral synaptic junctions, OA accelerated the release kinetics (Vyshedskiy *et al*., 1998) and increased transmitter release under low release conditions (Abdul-Ghani *et al*., 1991; Swain *et al*., 1991). In view of the profound regulations of membrane traffics and vesicle exocytotic cascade by protein kinase C and phosphoinositides (Vaughan *et al*., 1998; Corvera *et al*., 1999; Hong & Chang, 1999), it is probable that cellular phosphatases exert up- and down-grade modulations of vesicle exocytosis depending on the activities of protein kinases, the replenishment of vesicles and the types of neurons.

In brief, the dominant pharmacological effects of OA and cantharidin were on the mobilizations of synaptic vesicles and myoplasmic $Ca^{2+}$. The depressions of synaptic transmission and contractile function by the phosphatase inhibitors suggest the importance of constitutive phosphorylation and dephosphorylation of intraterminal components in the maintenance of motor function.

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