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Suramin inhibits the toxic effects of presynaptic neurotoxins at the mouse motor nerve terminals

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Abstract

Clinically available chemical antagonists of snake neurotoxins still await to be identified. In this study, we demonstrate that an anti-trypanosomiasis agent, suramin, is an effective inhibitor of β-bungarotoxin isolated from the venom of Formosan Krait snake. Following intraperitoneal injection (12 ng/g) of β-bungarotoxin in mice, the time to paralysis (loss a limb withdrawal reflex, 21.8 ± 3.4 h, $n = 4$) was significantly prolonged after intravenous injection (16 μg/g) of suramin (35.9 ± 4.0 h, $n = 4$, $P < 0.05$). The mechanism of this inhibitory effect of suramin was analyzed at the mouse nerve terminals. β-Bungarotoxin (1 μg/ml) produces an irreversible blocking effect of nerve-evoked muscle contractions of mouse phrenic nerve–diaphragm (blocking time 135 ± 6 min, $n = 6$). Pretreatment with suramin (0.3 mM) significantly prolonged the blocking time by three-fold. This selective inhibitory effect of suramin was further confirmed when suramin was shown to delay the neuromuscular blocking effect of another presynaptic neurotoxin, crotoxin (from American rattlesnake venom), but not that of the postsynaptic neurotoxin, α-bungarotoxin. Furthermore, suramin inhibited β-bungarotoxin in blocking transmitter release as revealed by prolonging the time to abolish the end-plate potential amplitude (with suramin, 391 ± 8 min; without treatment, 141 ± 5 min). K^+ current was measured in the mouse triangularis sterni preparation; suramin (0.3 mM) had no significant effect on β-bungarotoxin in inhibiting K^+ current ($77 \pm 3\%$ of control; with suramin $75 \pm 3\%$ of control, respectively). These findings clearly show that suramin is an inhibitor of presynaptic neurotoxins, mediated by interrupting the toxins in blocking the releasing mechanism of transmitter at the motor nerve terminals. The implication of these findings is that suramin and related compounds can become useful agents in management of snakebites.

Keywords: Suramin; β-Bungarotoxin; Nerve terminal; Transmitter release

1. Introduction

β-Bungarotoxin is a selective presynaptic neurotoxin isolated from the snake venom of Formosan krait, *Bungarus multicintus* (Chang et al., 1973). This neurotoxin consists of two polypeptides; an A chain possessing phospholipase A_2 activity and a B chain with protease inhibitor homology (Lee and Ho, 1982). It has been proposed that phospholipase A_2 activity of β-bungarotoxin is responsible for its neuromuscular blocking action (Chang, 1985). We have supported this contention by the finding that uranyl nitrate ($UO_2(NO_3)_2$) is a selective antagonist of β-bungarotoxin both in vitro and in vivo, probably by inhibiting phospholipase A_2 activity of β-bungarotoxin (Lin-Shiau and Fu, 1986; Lin-Shiau et al., 1984). In addition, Ueno and Rosenberg (1990) found that presynaptic toxins could inhibit phosphorylation of synaptosomal proteins but this finding in relation to the antagonism of transmitter release still remains to be elucidated.

Suramin has been used as a therapeutic agent in the treatment of trypanosomiasis. It also is an antagonist of P_2 purinoceptors (Dunn and Blakeley, 1988; Den Hertog et al., 1989; Nakazawa et al., 1991). Further studies showed that suramin not only reversed the effects of non-depolarizing neuromuscular blockers (Henning et al., 1992, 1993) but also inhibited the prejunctional Ca^{2+} channels (Henning et al., 1996). These effects of suramin at neuromuscular junctions seem to be irrelevant to its effects on P_2 purinoceptors.

Recently, we have demonstrated that suramin can completely reverse the curare-like action of a novel tripeptide,

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carbobenzoxy–Gly-Gly-Arg–β-naphthylamide, not only at the postsynaptic nicotinic acetylcholine receptors but also at the presynaptic autoreceptors (Lin-Shiau and Lin, 1998). In considering its effects on the presynaptic site of transmitter release, we proceeded to examine if suramin could exert its influence on the neuromuscular blocking effect of the presynaptic neurotoxin β-bungarotoxin. It turned out that suramin is an effective antagonist of β-bungarotoxin both in vitro and in vivo. The mode of action and possible action mechanism of suramin are studied in this paper.

2. Materials and methods

2.1. Mouse triangularis sternus preparation

Mice of ICR strain (18–25 g) were sacrificed by rapid cervical dislocation. The left triangularis sterni intercostal nerve–muscle preparation was isolated according to the method described previously (McArdle et al., 1981; Mallart, 1985; Lin and Lin-Shiau, 1997). The isolated preparations were pinned out on the sylgard-coated glass chamber (1–2 ml) and visualized at 320 × magnification by a Zeiss microscope (Axioskop) equipped with Nomarski interference contrast optics. Preparations were continuously perfused at a rate of 3–5 ml min^{-1} with oxygenated (95% O_2 plus 5% CO_2) modified Krebs solution containing (mM): NaCl 131, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 12.5 and glucose 11, pH 7.2–7.4. Experiments were performed at temperatures ranging from 24° to 28°C. The intercostal nerves were stimulated by a suction electrode using supramaximal voltage and square-wave pulses of 0.02 ms duration at 0.2 Hz (A-M systems model 2100 stimulator). Presynaptic waveforms were obtained from extracellular recording close to nerve terminal endings of intercostal nerves with glass microselectrodes filled with 2 M NaCl (resistance 5–15 MΩ) placed inside the perineural sheath (Mallart, 1985). Postysnaptic activity was blocked by adding 20–30 μM D-tubocurarine to the bathing medium. The signals of presynaptic waveforms were displayed on an oscilloscope (Tektronix 2221A) and stored in a videotape recorder (Neuro-Corder Recording Unit).

2.2. Mouse phrenic nerve–diaphragm preparation

Phrenic nerve–hemidiaphragm preparations were isolated and suspended in 5–10 ml of oxygenated modified Krebs solution. Chemicals were applied by perfusion. Miniature end-plate potentials (mepps) and end-plate potentials (epps) were measured by an intracellular glass microelectrode with a high impedance amplifier (Axoclamp 2A) in bridge mode. Microelectrodes were filled with 3 M KCl (resistance of 5–15 MΩ). Epps were evoked by stimulation of the phrenic nerve (fixed by bipolar suction electrode) at a frequency of 0.1 Hz with 0.02 ms supramaximal rectangular pulses (A-M systems model 2100 stimulator), and the diaphragm was immobilized by the cut muscle method (Barstad and Lilleheil, 1968).

The signals of epps, mepps and presynaptic current were displayed on an oscilloscope (Tektronix 2221A) and stored in a videotape recorder and then later played back for analysis. The signals were then later played back onto a waveform analyzer Data Precision (DATA 6000 with a Plug-In Model 610) for analysis and drawn by X–Y plotter or HC plotter (Tektronix).

2.3. In vivo studies

Both suramin and β-bungarotoxin were dissolved in 0.9% saline. Suramin (16 μg/g) was administered via tail vein intravenous injection 30 min before β-bungarotoxin (12 ng/g) via intraperitoneal injection in mice. The time to paralysis was recorded and compared with the group injected with β-bungarotoxin alone. The time to immobilization (paralysis) was assessed by loss a limb withdrawal reflex and dyspnea, then the mice were immediately sacrificed by gaseous carbon dioxide. This study was conducted in accordance with the guideline for the care and use of Laboratory Animals by the Animal Research Committee in National Taiwan University, College of Medicine. All mice were housed in the cage and maintained on 12-h light and dark cycle and allowed free access to food and water.

2.4. Statistics

The data are given as mean ± S.E.M. The significance of differences was evaluated by paired or unpaired Student's *t*-test. When more than one group was compared with one control, significance was evaluated using one-way analysis of variance (ANOVA). Probability values (P) of less than 0.05 were considered to indicate significance.

3. Results

3.1. Suramin delayed the neurotoxic effect of β-bungarotoxin in mice

Administration of β-bungarotoxin (12 ng/g) intraperitoneally produced a paralysis (loss a limb withdrawal reflex) of mice at 21.8 ± 3.4 h after the injection ($n = 4$).

Table 1
Suramin delayed the time to paralysis induced by β-bungarotoxin in the mice. Data are presented by mean ± S.E.M. Suramin was administered by intravenous injection to the mouse tail vein. β-Bungarotoxin was intraperitoneally injected. Both compounds were dissolved in 0.9% saline

Treatment	*n*	Time to paralysis (h)
β-Bungarotoxin (12 ng/g)	4	21.8 ± 3.4
Suramin (16 μg/g) plus β-bungarotoxin	4	35.9 ± 4.0[a]

[a] $P = 0.0363$ as compared to that of the β-bungarotoxin alone group.

Pretreatment with suramin (intravenous injection of 16 μg/g for 30 min) significantly prolonged the time to paralysis induced by β-bungarotoxin (35.9 ± 4.0 h, $n = 4$, $P = 0.0363$) (Table 1).

3.2. Suramin delayed the neurotoxic effect of β-bungarotoxin on nerve-evoked muscle contractions

The sustained treatment of mouse phrenic nerve–diaphragm with β-bungarotoxin (1 μg/ml) without washout produced an irreversible blocking effect on nerve-evoked muscle contractions at 135 ± 6 min (Fig. 1A, Table 2). Treatment with β-bungarotoxin for 20–30 min followed by washout of β-bungarotoxin completely blocked the neuromuscular transmission at 147 ± 7 min (Fig. 1C) which is not significantly different from that without washout of the toxin. This finding indicated that a 30-min treatment of β-bungarotoxin was sufficient to produce an irreversible neuromuscular blocking effect. Suramin (0.3 mM) treatment effectively delayed the blocking effect of β-bungarotoxin, as revealed by a marked prolonged time required for complete neuromuscular blockade from a control value of 135 ± 6 min to 390 ± 8 min (Fig. 1B; Table 2). Suramin application to the diaphragm pretreated with β-bungarotoxin for 30 min followed by washout of

Table 2
The blocking effect of neurotoxins on nerve-evoked muscle contractions of mouse phrenic nerve–diaphragm pretreated with or without suramin. Suramin was pretreated for 20 min prior to the application of neurotoxins. Data are presented as mean ± S.E.M.

Toxins	Concentration (μg/ml)	n	Time to complete blockade (min)
Without pretreatment (control)			
β-Bungarotoxin	1	6	135 ± 6
Crotoxin	3	3	150 ± 9
α-Bungarotoxin	1	3	30 ± 9
Pretreatment with suramin (0.3 mM)			
β-Bungarotoxin	1	6	390 ± 8[a]
Crotoxin	3	3	320 ± 12[a]
α-Bungarotoxin	1	3	33 ± 7

[a] $P < 0.001$ as compared with that of control without suramin.

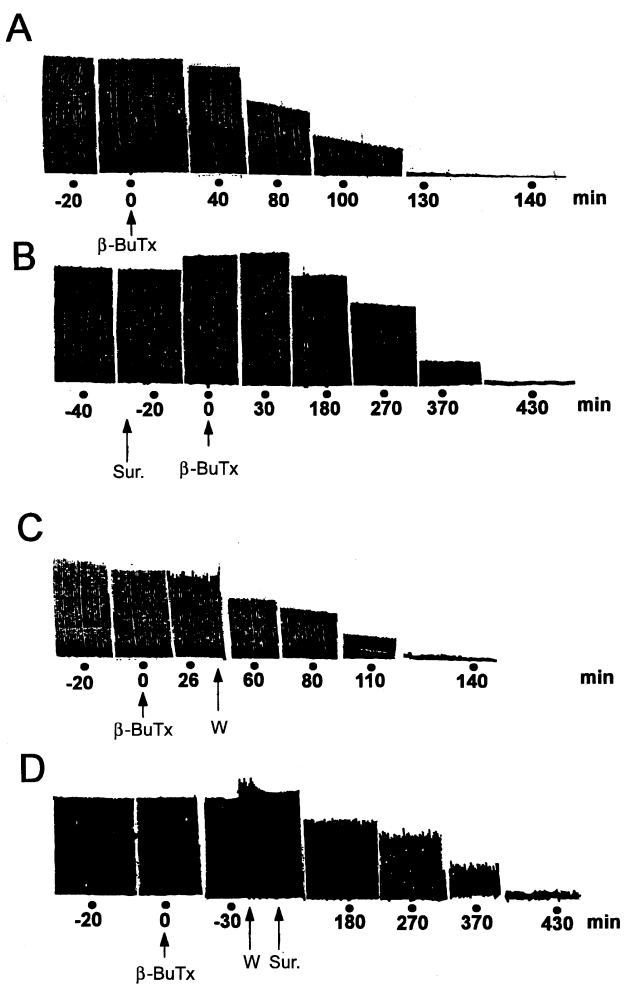

Fig. 1. Prolongation by suramin of the time required to complete neuromuscular blockade induced by β-bungarotoxin in the mouse phrenic nerve–diaphragm. Treatment with β-bungarotoxin (A, β-bungarotoxin 1 μg/ml) either without or with washout (W) after 20–30 min (C), produces an irreversible blockade of nerve-evoked muscle contractions. Pretreatment with suramin (0.3 mM) either prior to (B) or after toxin with washout (D) significantly prolongs the time required to blockade to three-fold of the control treated with β-bungarotoxin alone. Calibrations: 5 min; 2 g.

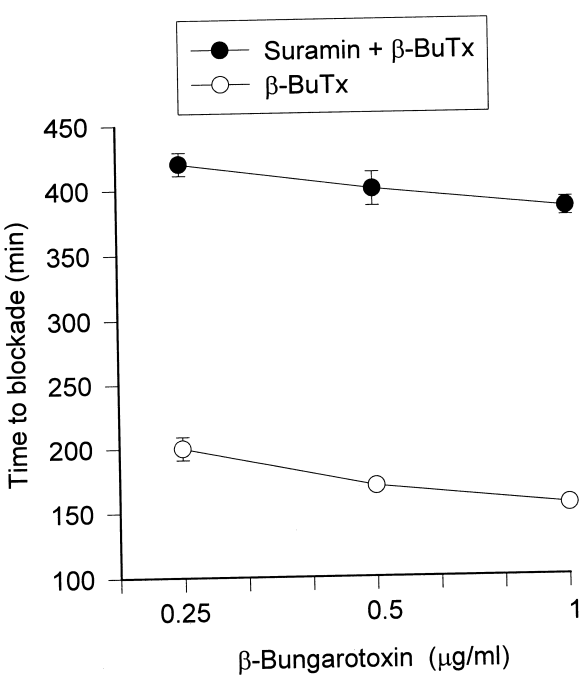

Fig. 2. Concentration-dependent blockade of nerve-evoked muscle contractions induced by β-bungarotoxin in the mouse phrenic nerve–diaphragm with or without suramin. Treatment with β-bungarotoxin alone (-○-) or with 0.3 mM suramin (-●-). Note that suramin markedly prolonged the time to complete blockade induced by β-bungarotoxin. From the extrapolation of concentration-time to blockade curve, the inhibitory potency of suramin estimated from these curves to be more than two-fold.

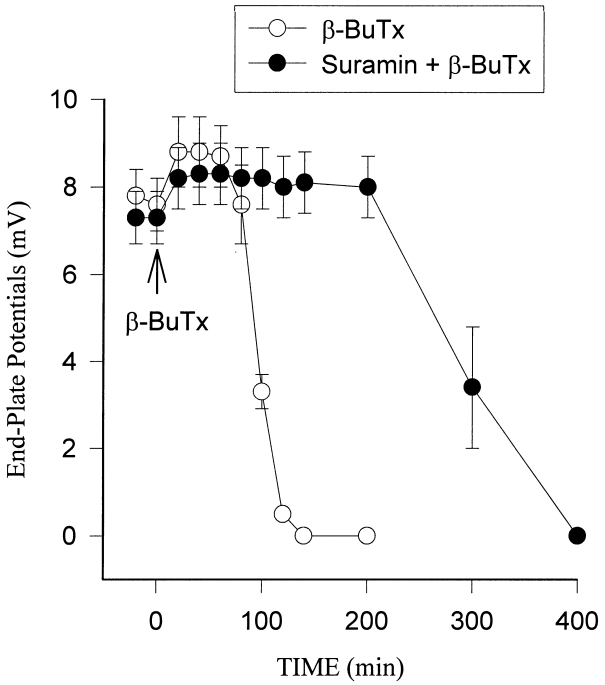

Fig. 3. Suramin prolonged the time required to blockade of epp induced by β-bungarotoxin in the mouse phrenic nerve–diaphragm. β-Bungarotoxin (1 μg/ml) produces an irreversible blocking effect of epp after 130 min (-○-) which is significantly prolonged to 400 min by pretreatment with 0.3 mM suramin (-●-).

β-bungarotoxin still exhibited a similar potent inhibitory effect (Fig. 1D). The inhibitory potency of suramin estimated from the concentration–response curve of β-bungarotoxin to be more than two-fold (Fig. 2). Further studies on the selectivity of this inhibitory effect of suramin indicated that suramin effectively inhibited crotoxin, another presynaptic neurotoxin by prolonging the blocking time from a control of 150 ± 9 min to 320 ± 12 min (Table 2). In contrast, suramin has no significant effect on postsynaptic neurotoxin, α-bungarotoxin (blocking time 33 ± 7 min and 30 ± 9 min with and without suramin, respectively) (Table 2).

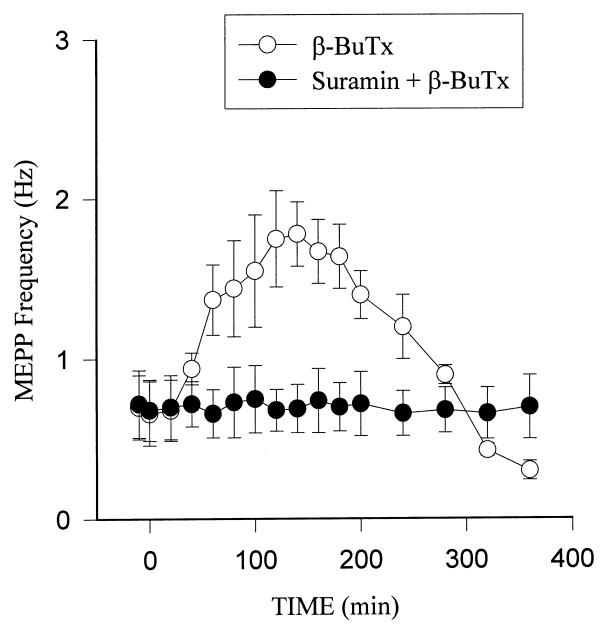

Fig. 4. Suramin abolishes the increased phase of mepp frequency induced by β-bungarotoxin in the mouse phrenic nerve–diaphragm. β-Bungarotoxin (1 μg/ml) increase mepp frequency within 50–100 min treatment (-○-). Suramin (0.3 mM)-pretreatment significantly attenuates the increased phase induced by β-bungarotoxin (-●-).

3.3. Suramin delayed the effects on epps and mepps

Similar to the effect of suramin on nerve-evoked muscle contractions, the time to complete blockade of epps by β-bungarotoxin (1 μg/ml) was prolonged by pretreatment of suramin (Fig. 3, blocking time 133 ± 12 min and 430 ± 15 min without and with 0.3 mM suramin, respectively). The increase of mepp frequency (Fig. 4) and amplitude (Fig. 5) induced by β-bungarotoxin was significantly attenuated by suramin as well. The amplitude histogram of mepp showed that suramin prevented the increased amplitude of mepps induced by β-bungarotoxin (Fig. 5).

3.4. Effect of suramin on nerve terminal K^+ current

We measured the K^+ current of triangularis sterni intercostal nerve terminal. β-Bungarotoxin (3 μg/ml)

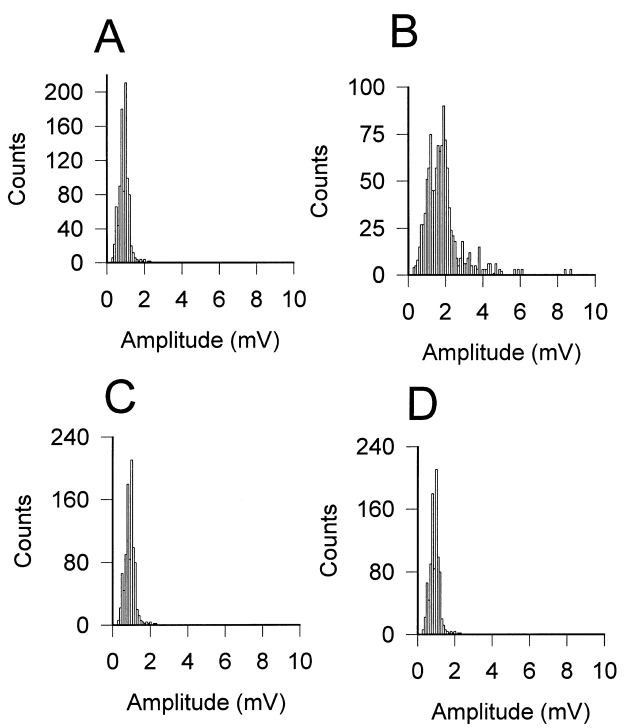

Fig. 5. Suramin abolishes the increased effect of mepp amplitude induced by β-bungarotoxin in the mouse phrenic nerve–diaphragm. The control and added β-bungarotoxin (1 μg/ml) for 80–90 min amplitude histogram were shown in A and B, respectively. Pretreatment with suramin (C, suramin alone) significantly attenuates the amplitude increasing effect induced by β-bungarotoxin (D).

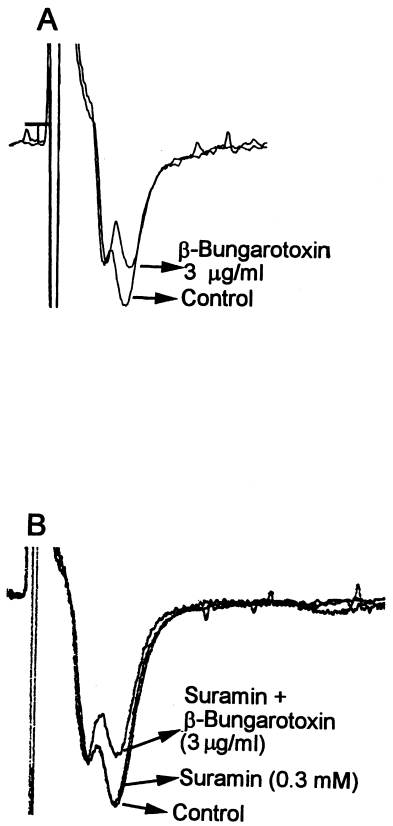

Fig. 6. Effect of suramin on the nerve terminal K^+ current inhibited by β-bungarotoxin in the mouse nerve–triangularis sterni. β-Bungarotoxin (3 μg/ml) inhibits the K^+ current to 77±3% of control (A) which is similar to that induced in the preparations pretreated with suramin (0.3 mM, 75±3%) for 15–20 min. Calibrations: 1 ms, 1 mV.

treatment for 15–20 min significantly inhibited the fast K^+ current to 77 ± 3% of control ($n = 4$). Pretreatment with suramin (0.3 mM) did not alter the effect of β-bungarotoxin on K^+ current (75 ± 3% of control, $n = 4$) (Fig. 6).

4. Discussion

In this paper, we are the first to demonstrate that suramin significantly inhibits the β-bungarotoxin in blocking the neuromuscular transmission both in vivo and in vitro. Suramin not only prolonged the time required for complete blockade on the nerve-evoked muscle contractions but also on that of epps of the mouse diaphragm. In addition, suramin inhibited the increased mepp frequency induced by β-bungarotoxin. Most importantly, suramin was shown to be capable of reducing the toxicity of β-bungarotoxin in mice in vivo. Because suramin exhibited a similar delayed effect either applied simultaneously with β-bungarotoxin or applied after accomplishment of the binding of β-bungarotoxin for 30 min followed by washout, this effect of suramin was considered to be mediated by interrupting β-bungarotoxin to inhibit the release mechanism of transmitter at the motor nerve terminals. Furthermore, suramin did not affect the inhibitory effect of β-bungarotoxin on the K^+ current. It is inferred that suramin is somehow specific to interfere with the binding process relative to the release mechanism rather than the membrane K^+ channels. The processes of the transmitter release mechanism such as the role of synapsin I and synaptic transmission have been investigated extensively. The phosphorylation of synapsin I causes the dissociation of synaptic vesicle from actin filaments leading to the movement of synaptic vesicles to the nerve terminals followed by docking and fusing with the nerve plasma membrane prior to the exocytosis of transmitter release (Schiebler et al., 1986; Sollner et al., 1993). Although the actual molecular mechanism of β-bungarotoxin in blocking transmitter release is still not known, it has been postulated that phospholipase A_2 activity of β-bungarotoxin plays a role in inhibiting acetylcholine release (Chang, 1985). However, Chapell and Rosenberg (1996) pointed out that the inhibitory effect of β-bungarotoxin was probably due to the interruption of the upstream transport of synaptic vesicles toward the active zone of nerve terminals rather than the membrane fusion processes between synaptic vesicle and the nerve terminal membrane. Since suramin inhibited the neurotoxic effects of β-bunagrotoxin and crotoxin, two phospholipase A_2 presynaptic neurotoxins, the possible mechanism of suramin mediated by interfering the phospholipase A_2 activity cannot be ruled out.

Suramin is a polysulfate anionic compound which is capable of reversing the neuromuscular blocking actions of the non-depolarizing muscle relaxant such as D-tubocurarine, but it is different from D-tubocurarine in that suramin by itself at a high concentration of 0.3 mM cannot inhibit the nerve-evoked muscle contraction. Recently, we have demonstrated that suramin completely reversed the curare-like action of a novel tripeptide, carbobenzoxy–Gly-Gly-Arg–β-naphthylamide. This tripeptide exerted its effects not only at the postsynaptic nicotinic acetylcholine receptors but also at the presynaptic autoreceptors (Lin-Shiau and Lin, 1998). Moreover, suramin can either depress the Ca^{2+} current at nerve terminals (Henning et al., 1996) or inhibit the postsynaptic ACh receptor. However, the calcium channel blocker or acetylcholine receptor blocker (e.g., Cd^{2+} or D-tubocurarine, respectively) did not alter the neurotoxic effect induced by β-bungarotoxin. Thus, we can infer that suramin has some effects on β-bungarotoxin irrelevant to its effect on calcium channel on ACh receptor blockade. The molecular mechanism of suramin on β-bungarotoxin is currently investigated. We are considering the possibility that β-bungarotoxin blocks transmitter release by a process related to phospholipase A_2, Ca^{2+}-transport and/or protein phosphorylation. This speculation needs further studies for clarification.

In conclusion, we are the first to report that suramin significantly inhibits the neuromuscular blocking effect of the presynaptic neurotoxins, β-bungarotoxin and crotoxin either in vivo or in vitro. This effect of suramin is specific since it has no effect on postsynaptic α-bungarotoxin. It is believed that suramin may provide a useful tool for further studies on the molecular mechanism of actions of β-bungarotoxin. Moreover, the effective delayed effect by suramin in vivo implicates that suramin could be a useful drug for management of the snakebite (e.g., Formosan krait).

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