Secreted phospholipases A₂ (sPLA₂s) are pharmacologically important components of several animal venoms. Their neurotoxic action on presynaptic neurons involves a reduction in endocytosis, leading to synaptic vesicle depletion. This results in suppression of the release of acetylcholine from neurons into the synaptic cleft, causing irreversible blockade of neurotransmission to the postsynaptic muscle cell. We have recently proposed a model of the molecular mechanism of action of the neurotoxic sPLA₂, ammodytoxin A (AtxA). It is based on the experimental demonstration that the AtxA-induced reduction in endocytosis is a consequence of: (1) the specific intracellular interaction of AtxA with 14-3-3 proteins, which enables the toxin molecules to concentrate on the plasma membrane near the endocytic vesicles; and of (2) the PLA₂ enzymatic activity of AtxA, which changes the shape and/or composition of the presynaptic membrane, thus inhibiting the activity of the endocytic protein amphiphysin. The study was, however, performed in yeast, making the validity of the proposed model for mammalian cells a pivotal question.

The reduction of endocytosis in yeast was not observed with the catalytically inactive structural paralog of AtxA, ammodytin L (AtnL). Here we confirm the validity of the model in mammalian cells also, by demonstrating that the enzymatically active mutant of AtnL, shown to inhibit endocytosis in yeast, acts as a presynaptically neurotoxic sPLA₂ at the mammalian neuromuscular junction.
had only a minor inhibitory effect on the twitch tension of a partly paralyzed NM preparation (data not shown). In experiments performed in standard Krebs solution with 2.5 mM Ca\(^{2+}\), in which the majority of motor end-plates within the NM preparation are sensitive to neuronal stimulation, AtxA inhibited muscle contractions in a manner consistent with our previous results.\(^7\) However, muscle contraction in standard Krebs solution was not affected by the addition of AtnL, at least not for up to 90 min, in the case of either indirect (Fig. 1C) or direct (Fig. 1D) stimulation of the muscle. This indicates that AtnL had no effect on acetylcholine release from the presynaptic neurons or on the functionality of the postsynaptic muscle cell. Furthermore, addition of a very potent presynaptically neurotoxic sPLA\(_2\), taipoxin, to an NM preparation pre-treated with AtnL, triggered the characteristic triphasic change of muscle contractility, demonstrating that this preparation retained the ability to respond to neurotoxic sPLA\(_2\)s (data not shown). These results show clearly that, by switching on calcium-binding and thus the PLA\(_2\) activity of AtnL, by replacing amino acid residues at four positions,\(^3\) this presynaptically inactive protein has been converted into a presynaptically active sPLA\(_2\) in mammalian cells.

Figure 1. AtxA and the LW mutant, but not the enzymatically inactive AtnL, display the triphasic modulation of twitch tension characteristic of presynaptically neurotoxic sPLA\(_2\)s. The effects of recombinant AtxA (10 \(\mu\)g/ml; 0.725 \(\mu\)M), the LW mutant (6 \(\mu\)g/ml; 0.430 \(\mu\)M) and AtnL (6 \(\mu\)g/ml; 0.433 \(\mu\)M) on muscle twitch tension were determined at 37°C. (A and B) The NM preparation was partially paralyzed by a low Ca\(^{2+}\) concentration (0.38–0.50 mM) Krebs solution and stimulated indirectly. Note that, upon addition of LW, the triphasic muscle twitch response occurred within 10 min (B). (C and D) The NM preparation was stimulated indirectly (C) or directly (D) in standard Krebs solution containing 2.5 mM Ca\(^{2+}\) as described in materials and methods. Note that AtxA, but not AtnL (C), inhibited muscle contractions. Proteins were added to the bath at time 0. Twitch tension is expressed as a percentage of the control value. The number of repeats of experiments is shown in brackets. Each point on the diagrams represents the mean ± SE. Standard errors are indicated by bars unless smaller than symbols.

A hallmark of the action of presynaptically neurotoxic sPLA\(_2\)s, including AtxA (Fig. 1A), is a triphasic effect on indirectly elicited muscle twitch tension of partially paralyzed neuromuscular (NM) preparations.\(^5,6\) The modulation of twitch tension induced by the LW mutant conformed to the characteristic triphasic action of presynaptically neurotoxic sPLA\(_2\)s—a short initial depression of muscle contraction followed by a transient increase in twitch tension and, third, a progressive decline in muscle contractility within 10 min after the application of the mutant (Fig. 1B). The triphasic effect occurred almost 10-fold faster than in the case of AtxA. In contrast, recombinant AtnL did not trigger the triphasic response and possibility that, with this acquired enzymatic activity, the LW mutant has also acquired the ability to act as a presynaptically neurotoxic sPLA\(_2\). The fact that the LW mutant and AtxA, but not AtnL, reduced endocytosis in the yeast model further strengthened such an explanation. To test the hypothesis that the mutations in AtnL that endowed it with PLA\(_2\) activity and the ability to impair endocytosis in yeast would also render it neurotoxic for mammals, we determined the effect of recombinant AtnL and its LW mutant on an isolated mouse phrenic nerve-hemidiaphragm preparation.
The progressive decline of NM activity, along with other characteristic morphological changes induced by presynaptically neurotoxic sPLA₂s at the NM junction (i.e., appearance of omega-shaped invaginations in the plasma membrane and synaptic vesicle depletion), has been ascribed to an irreversible blockade of synaptic vesicle retrieval from the plasma membrane. This parallels the impairment of the endocytic machinery observed on expression of AtxA and LW in yeast cells. To summarize, the reduction in endocytosis previously observed in yeast cells, coupled with the neurotoxic action at the mouse NM junction by AtxA and the enzymatically active mutant of AtnL, but not by AtnL itself, confirm that the proposed model of sPLA₂ toxicity in yeast is applicable to the molecular mechanism of action of neurotoxic sPLA₂s in mammals.

Materials and Methods

Recombinant proteins and chemicals. Recombinant AtxA, AtnL and its quadruple H28Y/L31V/N33G/S49D mutant LW were prepared by in vitro refolding following expression in E. coli as described. All other chemicals were from Sigma Aldrich or Gibco Life Technologies.

Neuromuscular activity. Adult male BALB/c mice (20–25 g) were maintained and humanely killed according to the Guiding Principles in the Use of Animals in Toxicology (Society of Toxicology, 1999, Guidelines are available at www.toxicology.org) and the Animals (Scientific Procedures) Act 1986. Hemidiaphragms and accompanying phrenic nerves were dissected and placed into 10 ml tissue baths containing Krebs solution (118.4 mM NaCl, 4.7 mM KCl, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.4 mM MgSO₄ and 2.5 mM CaCl₂), maintained at 37°C and oxygenated with a mixture 95% O₂ and 5% CO₂, and gassed to do 118.4 mM NaCl, 4.7 mM KCl, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.4 mM MgSO₄ and 2.5 mM CaCl₂), maintained at 37°C and oxygenated with a mixture 95% O₂ and 5% CO₂. Muscle contractions were evoked indirectly or directly. In the case of indirect neurally-evoked twitches, the attached phrenic muscle near the costal margin and the other one was attached to the base of the hemidiaphragm. The directly-evoked twitches were recorded by supramaximal voltage stimulation (50 V) at a frequency of 0.2 Hz and 2 ms duration. To eliminate NM stimulation in the preparation, d-tubocurarine (10 μM), an antagonist of the nicotinic acetylcholine receptors, was added into the organ bath 5 min prior to the beginning of direct stimulation. Positive controls for the neurotoxic triphasic effect were performed by adding taipoxin (0.5–1 μg/ml) after reducing the concentration of Ca²⁺ in order to partially paralyze the NM preparation as described above.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We sincerely thank Dr. Roger H. Pain for critical reading of the manuscript. This work was supported by grants P1-0207, Z1-4468 and J1-6507 from the Slovenian Research Agency and by NATO Collaborative Linkage Grant No. 980899.

References
1. Pungerčar J, Križaj I. Understanding the molecular mechanism underlying the presynaptic toxicity of secreted phospholipases A₂. Toxicon 2007; 50:871-92; PMID:17905401; http://dx.doi.org/10.1016/j.toxicon.2007.07.025.
2. Martszzi M, Sun Y, Wolinski H, Bavek A, Petan T, Anderluh G, et al. A neurotoxic phospholipase A₂ impairs yeast amphiphysin activity and reduces endocytosis. PLoS ONE 2012; 7:e40931; PMID:22844417; http://dx.doi.org/10.1371/journal.pone.0040931.
3. Petan T, Križaj I, Pungerčar J. Restoration of enzymatic activity in a Ser-49 phospholipase A₂ homologue decreases its Ca²⁺-independent membrane-damaging activity and increases its toxicity. Biochemistry 2007; 46:12795-809; PMID:17927217; http://dx.doi.org/10.1021/bi0701304e.
4. Lomonte B, Rangel J. Snake venom Lys49 myotoxins: From phospholipases A₂ to non-enzymatic membrane disruptors. Toxicon 2012; 60:520-30; PMID:22781132; http://dx.doi.org/10.1016/j.toxicon.2012.02.007.
5. Lee CY, Tsai MC, Chen YM, Ritonja A, Gubeniček F. Mode of neuromuscular blocking action of toxic phospholipases A₂ from V. ammodytes venom. Arch Int Pharmacodyn Ther 1984; 268:313-24; PMID:6732682.
6. Prijatelj P, Vardjan N, Rowan EG, Križaj I, Pungerčar J. Binding to the high-affinity M-type receptor for secreted phospholipases A₂ is not obligatory for the presynaptic neurotoxicity of ammodytoxin A. Biochimie 2006; 88:1425-33; PMID:16815622; http://dx.doi.org/10.1016/j.bioch.2006.06.008.
7. Logonder U, Križaj I, Rowan EG, Harris JB. Neurotoxicity of ammodytoxin A in the envenoming bites of Vipera ammodytes ammodytes. J Neuropathol Exp Neurol 2008; 67:1011-9; PMID:18800066; http://dx.doi.org/10.1097/NEN.0b013e318188c2d7.
8. Dixon RW, Harris JB. Nerve terminal damage by beta-bungarotoxin: its clinical significance. Am J Pathol 1999; 154:447-55; PMID:10027403; http://dx.doi.org/10.1016/S0002-9440(10)62529-1.
9. Petan T, Križaj I, Gelb MH, Pungerčar J. AtnL itself, confirm that the proposed model of sPLA₂ toxicity in yeast is applicable to the molecular mechanism of action of neurotoxic sPLA₂s in mammals.